The effect of antidepressant drugs on the circadian rhythm of 5-hydroxytryptamine synthesis in the CNS

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THE EFFECT OF ANTIDEPRESSANT DRUGS ON THE CIRCADIAN RHYTHM OF 5-HYDROXYTRYPTAMINE SYNTHESIS IN THE CENTRAL NERVOUS SYSTEM

Submitted by

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For my mother

and the loving memory of my father

dearly departed.

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

Sir William Lawrence Bragg 1890-1971

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Abstract

This study investigates the effects of antidepressant drugs on the circadian rhythm of 5HT synthesis in the rat brain. In Chapter One, the reader is familiarized with the relevant subjects that are covered later in the thesis, viz., neurochemistry of the serotonergic system, physiology of the circadian rhythms and physiology and biochemistry of affective disorders.

The activity of tryptophan hydroxylase was assayed by both *in vitro* and *in vivo* techniques. The results revealed the existence of a significant circadian rhythm in its activity and hence, presumably, in the rate of 5HT synthesis. Though the results indicate that this rhythm is nearly 180^{o} out of phase with that of the concentration of 5HT, the possibility is considered that it may still be at least partly involved in regulating the circadian rhythm of 5HT concentration. After an extensive literature review, it is proposed that the circadian rhythm of tryptophan hydroxylase activity is in turn probably regulated by that of the spontaneous firing rate of the serotonergic neurons.

Acute and chronic treatment with different classes of antidepressant drugs had marked though not necessarily similar effects on the nadir and zenith of the circadian rhythm of 5HT synthesis. These results suggest that the serotonergic system may be an important locus of action of antidepressant drugs.

Aims

It is now more or less firmly established that the concentration of 5HT in the brains of laboratory animals displays a marked circadian rhythm. What is not clear are the factors and the mechanism for generating the rhythm. The activity of tryptophan hydroxylase, the rate limiting enzyme in its synthesis, has been suggested as a possible candidate. For this argument to be tenable, it would be necessary to demonstrate a circadian rhythm in the activity tryptophan hydroxylase synchronized with that of the 5HT concentration. It is a matter of debate whether this enzyme has a circadian rhythm in its activity because both negative and positive results have been reported independently in different laboratories. In view of this controversy, experiments were undertaken in this study to investigate this problem further.

An attempt was also made to determine the effects of acute and chronic treatment with selected antidepressant drugs on the circadian rhythm of 5HT synthesis. This was done mainly with two objectives in mind. As concluded from the literature survey, there is a lack of consensus on the effects of antidepressants on the serotonergic neurons and on the mechanism of action of these drugs. Since the regulation of 5HT synthesis is one major mode of modulating the activity of serotonergic system, it was hoped that an examination of its effects by antidepressants might provide an insight into the regulatory mechanisms involved. Secondly, abnormal circadian rhythms are associated with affective disorders and it has been postulated that the efficacy of antidepressant drugs is due to their ability to interact with and restore the circadian system to a normal equilibrium. The response of the circadian rhythm of 5HT synthesis

to both acute and chronic antidepressant administration was therefore examined as one approach of determining the mechanism of action of these drugs.

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Chapter 1

INTRODUCTION

1.1. SEROTONIN IN THE CENTRAL NERVOUS SYSTEM

1.1.1. Historical introduction

In the middle of the nineteeth century, a vasoconstrictor substance was discovered in serum. The nature of this substance was to remain unknown for nearly a century. In 1932 Bayliss and Ogden found that "vasotonins" could be removed from serum by perfusion through lungs.

Another line of investigation was concerned with intestinal contractile action of these substances. In 1933 Vialle and Erspamer gave the name "enteramine" to a vasoconstrictor substance or substances contained in the extract of gastric mucosa. It was believed to originate in the "enterochromaffin" cells of gastro-intestinal tract. They also demonstrated its existence in a number of mammalian tissues. Its chemical nature, however, was uncertain nor was it isolated.

It was not until the late 1940s however, that the vasoconstrictor substance was isolated and characterised by Page and his colleagues. They named it "serotonin". It proved to be 5-hydroxytryptamine (5HT), which was soon synthesised and characterised. Shortly afterwards "enteramine" and serotonin were shown to be identical. Thus by the time serotonin was identified it was known to be a normal constituent of several mammalian tissues.

1.1.2. Occurrence of 5HT

The occurrence of serotonin is not unique to mammals. It is widely distributed in both plant and animal kingdoms. It occurs in coelenterates, anthropods, molluscs, tunicates and all vertebrate classes. It is also found in such fruits as bananas, pineapples,

plums, nuts and in various venoms.

1.1.3. Distribution of 5HT in mammalian central nervous system

The central nervous system contains only about one percent of the total serotonin in the body. It is unevenly distributed in the brain. The highest concentrations are found in the hypothalamus, midbrain and brainstem, followed by cerebral cortex, hippocampus and striatum. Cerebellum has the lowest concentration.

The details of the regional distribution emerged only after the development of histochemical fluorescence techniques of Falck and his colleagues (1962, quoted by Green and Graham-Smith, 1975). In this method, serotonin is converted into a fluorophore by exposure to formaldehyde. Serotonin containing neurons can then be visualized directly. Unfortunately, the fluorescence technique for 5HT is not as sensitive as that for catecholamines. Furthermore, the fluorescence is very sensitive to ultraviolet light and fades within seconds, especially in small terminals. However, it has proved useful especially when combined with pharmacological manipulation, for example, pre-treatment with MAO inhibitors and a large dose of tryptophan to increase 5HT levels.

Newer techniques more suitable for mapping discrete projections have recently been developed. These include: immunocytochemical localization of tryptophan hydroxylase and 5HT; orthograde axoplasmic transport of radio-labelled amino acids microinjected into cells identified as containing 5HT by fluoresence histochemistry; retrograde transport of horse-radish peroxidase and autoradiographic localization of tritiated serotonin. These techniques have been used

extensively to map serotonergic and other neurotransmitter pathways in the brain.

Most of the serotonin in the brain is synthesised in the neurons mainly concentrated in raphe nuclei located in the brainstem and mesencephalon. From here descending and ascending axons project to provide 5HT containing nerve endings in other areas of the brain and the spinal cord. For example, the more rostrally placed neurons of the raphae nuclei innervate telencephalon and diencephalon, while the caudally placed cells send processess to the medulla and the spinal cord. Neurons in the median raphae nucleus project to limbic structures; neurons in the dorsal raphae project primarily to the neostriatum, cerebral cortex, thalamus and the cerebellum. These and other pathways and how they were identified are described in detail by Dalstrom et al., (1973).

There is evidence, however, that not all serotonin in the brain is synthesized in the raphe nuclei. For example, complete ablation of the medial basal hypothalamus depletes only about 70% of its serotonin (Brownstein et al., 1976). The rest is presumably synthesized locally.

1.1.4. Biosynthesis and metabolism of serotonin

Brain cells must synthesize their own 5HT because it cannot cross the blood-brain barrier from the peripheral circulation. It is synthesized from tryptophan, an essential amino acid obtained primarily from the diet.

The first step in the synthesis of 5HT involve the uptake of tryptophan into the neurons. The uptake is an active process and is open to competition by other large neutral amino acids that share the

same transport system such as the aromatic amino acids tyrosine and phenylalanine, the branched chain amino acids leucine, isoleucine and valine.

The next step is hydroxylation of tryptophan at the 5-position of the indole ring to form 5-hydroxytryptophan (fig. 1). This reaction is the rate limiting step in the synthetic pathway. It is catalysed by tryptophan hydroxylase, a mixed-function oxidase which requires molecular oxygen and a pteridine cofactor, tetrahydrobiopterin. Once synthesized, 5-hydroxytryptophan is almost immediately decarboxylated by the enzyme 5-hydroxytryptophan decarboxylase to form serotonin. The mechanisms and factors involved in the control of the synthesis of serotonin are examined in more detail in Chapter 2.

The most important enzyme in catabolism of 5HT is monoamine oxidase (MAO), which oxidizes the amino group to form an aldehyde, 5-hydroxyindoleacetaldehyde. The aldehyde is rapidly oxidized further to yield 5-hydroxyindoleacetic acid (5HIAA). It could also be reduced to form an alcohol, 5-hydroxytryptophol, depending on the NAD^+/NADH ratio in the tissues. MAO, which is located on the outer surface of the mitochondria, exists in two forms, A and B. Type A is more sensitive to inhibition by clorgyline and its substrates are tyramine and 5HT. Type B is inhibited to a greater extent by deprenyl than by clorgyline and it oxidizes tyramine well, but not 5HT. The two forms are distributed differentially in the rat brain (Neff and Goridis, 1972, quoted by Green and Constain, 1981) and this led to a suggestion of physiological significance. However, this view has not been supported by the observation that MAO in human brain is almost exclusively type B (Glover et al., 1977).

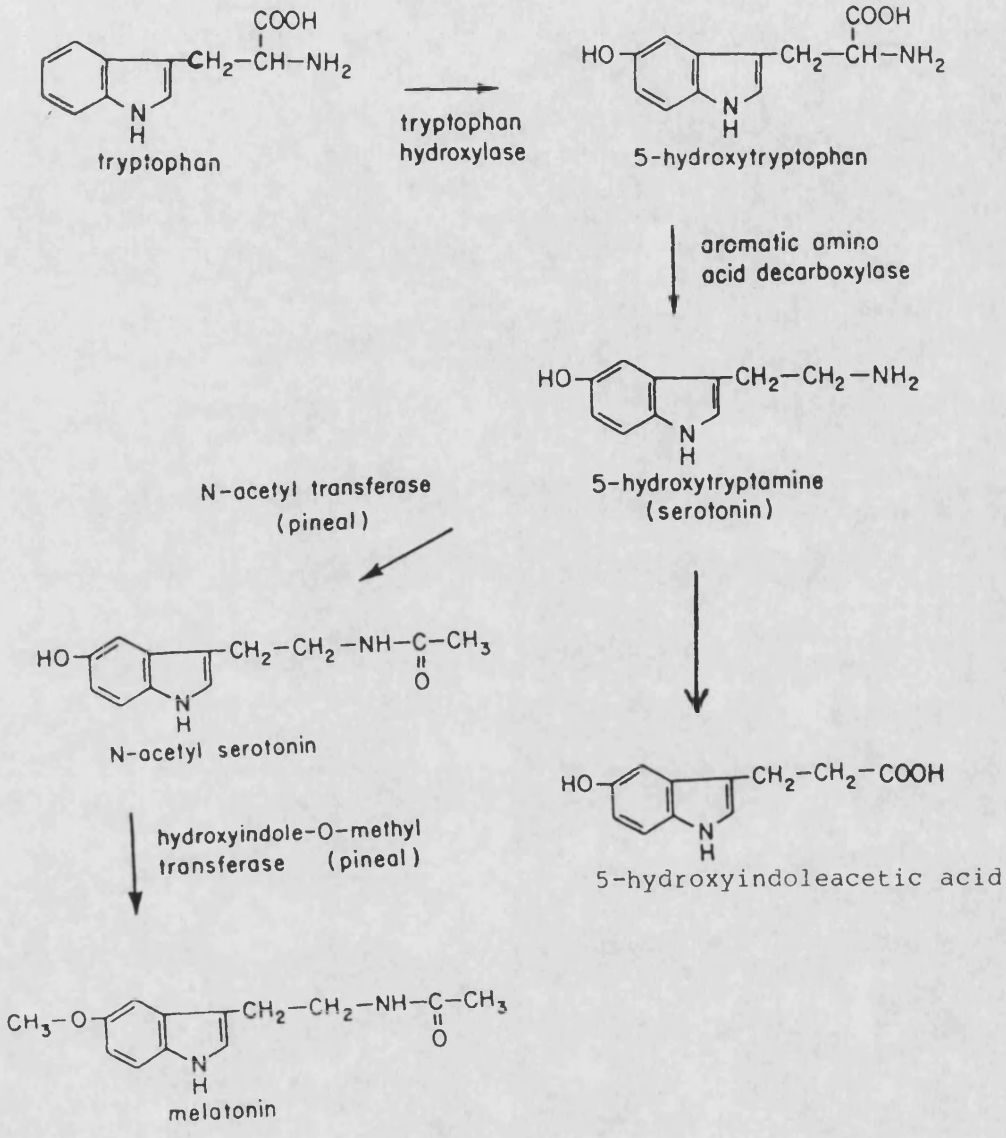


Fig. 1. Biosynthesis and metabolism of 5-Hydroxytryptamine(Serotonin).

The brain contains 5HT-sulfotransferase enzyme which, using sulfate-donating properties of 3'-phosphoadenosine 5'-phosphosulfate, converts 5HT to 5HT-o-sulfate. Though this is an important pathway for 5HT inactivation in the periphery, its significance in the brain has been questioned (Green and Graham-Smith, 1975).

Serotonin is converted to melatonin in the pineal gland. It is first N-acetylated by 5HT-N-acetyltransferase to form N-acetylserotonin, which is then methylated. This step requires the enzyme 5-hydroxyindole-o-methyl transferase, utilising S-adenosyl methionine as the methyl donor. The product is melatonin, which is then secreted into the systemic circulation. Melatonin secretion follows a cyclic daily rhythm with the highest concentration in the dark. This rhythm is controlled by the suprachiasmatic nucleus in the hypothalamus. It acts through the sympathetic system by influencing 5HT N-acetyltransferase whose activity rhythm follows the same phase as melatonin. The activation of this enzyme is mediated by cyclic-AMP via β-receptors (see also section 1.1.8.).

1.1.5. Storage, release and inactivation of 5HT

Knowledge about regulation of serotonin storage and release have been obtained mainly by <u>in vitro</u> studies using tissue slices or partially purified synaptosomes from brain areas known to be rich in serotonin nerve endings. Various <u>in vivo</u> methods are also available. Serotonin and other neurotransmitters released from nerve terminals surrounding the ventricles can be collected using cerebroventricular perfusion. Push-pull cannulae have been developed to measure transmitters released in discrete brain areas. Voltammetry or electrochemical recording is a promising novel technique. In this

technique, micrographic electrodes are 'implanted' into selected brain areas to measure minute electrical currents resulting from oxidation or reduction of electroactive compounds. The potential at which the reaction occurs indicates the nature of the compound while the amount of the current is proportional to its concentration.

It is believed that serotonin is stored primarily in synaptic vesicles where it is protected from deamination by monoamine oxidase. Two storage pools have been suggested; the small and readily releaseable "functional" and the large and relatively stable "reserve" pools (Glowinski, 1975; Morot-Gaudry et al., 1981). It appears that one of these pools represents a soluble form of storage (Halaris and Freedman, 1977; Tamir and Huang, 1974). A specific serotonin binding protein associated with synaptic vesicles has been identified (Tamir and Gershon, 1979).

In the platelet, serotonin is stored in specific cytoplasmic organelles with dense osmiophilic cores. As in the brain, a second storage site exists in the platelet. However, the specific serotonin binding protein differs in physical and chemical characteristics from that in the brain (Tamir et al., 1980).

Evidence suggest that serotonin is released by a process of exocytosis, though the mechanism is largely unknown (Kuhn, Wolf and Youdim, 1986). Studies show that newly synthesized serotonin is preferentially released during depolarization by a Ca^{2+}-dependent process. Depolarization of brain slices or synaptosomes induces influx of Ca^{2+} into the cell. This stimulates fusion of vesicular membrane into plasma membrane followed by simultaneous release of serotonin and serotonin-binding protein. The process of release is

believed to be mediated by cyclic AMP-dependent and Ca^{2+}-dependent protein kinases through phosphorylation of protein I (Sanders-Bush and Martin, 1982).

Serotonin, like other neurotransmitters is able to modulate its own release. This effect is mediated by autoreceptors located on pre-synaptic nerve terminals. Binding of serotonin to the autoreceptors (due to increased concentration at the synaptic cleft) inhibits its own release (Sanders-Bush and Martin, 1982).

The serotonergic cells in the raphé nuclei show a spontaneous electrical activity. It is well documented that release of serotonin at the nerve terminals is dependent upon this firing rate. Electrical stimulation of these cells increase serotonin release at the nerve endings and hence its metabolism to 5HIAA. When 5HT is administered microelectrophoretically to the raphé nuclei cells, the majority of them decrease their discharge rate (Aghajanian and Wang, 1978). Increase of serotonin in the nerve endings caused by, say, increased synthesis or decreased re-uptake leads to a depression of the firing rate followed by decreased release and synthesis. This observation led to the suggestion that other autoreceptors are also located in the cell bodies in the raphé nuclei and that they receive inputs from post-synaptic interneuronal feedback loops (reviewed by Aghajanian and Wang, 1978).

There is evidence that other neurotransmitters and co-transmitters also play part in the regulation of serotonin release. Several studies have shown for example, that noradrenaline inhibits serotonin release by acting through α_2-receptors located in the serotonergic terminals (reviewed by Sanders-Bush, 1982).

Monoamine oxidase is believed to be involved in the

physiological regulation of serotonin storage and release (Green and Graham-Smith, 1975). This is supported by both *in vivo* and *in vitro* studies which demonstrate that tryptophan loading increases serotonin synthesis but not release. Pre-treatment with a monoamine oxidase inhibitor on the other hand increases both synthesis and release (Marsden et al., 1979; Elks, Youngblood and Kizer, 1979). Clearly then, increase in serotonin synthesis is not necessarily followed by an increase in release since excess serotonin can be metabolized by MAO intraneuronally without being released (Kuhn, Wolf and Youdim, 1986).

Serotonin is mainly inactivated at the synaptic cleft by re-uptake into pre-synaptic nerve endings where it is degraded intraneuronally by MAO (Iversen, 1975). Some of it may be recaptured into the storage granules.

1.1.6. Serotonin Receptors in the CNS

As early as 1878 the concept of drug-receptor interaction was introduced by Langley. From his observation that some drugs were highly specific in mimicking a response while others prevented it, he suggested that they interacted with receptors to elicit the pharmacological response. In the intervening period of about a century, receptors have been identified for neurotransmitters, peptides and steroid hormones etc. Some have been isolated as macromolecular substances. All the receptors for neurotransmitters that have been studied are found to be located on the surface of the cell.

A receptor has been defined by Hollenberg and Cuatrecasas (1978,

quoted by Sulser and Janowsky, 1982) as a recognition site whose binding generates a signal linked to a biological response. This distinguishes it from an acceptor, defined as a binding site for a ligand which does not lead to a biological response no matter how high its affinity and specificity is. This distinction became particularly important in view of the likely confusion brought about by the multiplicity of binding sites encountered, especially after the introduction of radiolabelled ligands.

As explained earlier, apart from those located on the post-synaptic membranes, serotonin receptors are also located at the presynaptic membranes and on the cell bodies of serotonin containing neurons in the raphé nuclei. The last two are termed autoreceptors and modulate the release of serotonin by feedback mechanism as discussed earlier. There is currently no evidence for the existence of serotonin autoreceptors in the periphery. The characteristics and pharmacology of serotonin autoreceptors have been reviewed in detail by Moret (1985).

Until recently the only means of studying interaction of hormones, neurotransmitters or drugs with receptors was based on observations of pharmacological or physiological effects of isolated organs to applied agonists or antagonists. A new and wider scope of study was opened by the development of highly specific radioactive ligands in the early 1970s. It has led to direct investigation of drug-receptor interactions, receptor localization and receptor regulatory mechanisms.

Lysergic acid diethylamide (LSD) is known to be an agonist at the serotonin receptor sites in the raphé nuclei, though it can act both as agonist and antagonist at the nerve terminals. Radio-

labelled [H^3]-LSD was the first ligand to be used for serotonin binding studies. Using [H^3]-LSD as well as labelled serotonin ([H^3]-5HT), Bennett and Snyder in 1976 demonstrated differences in the number, affinity and regional distribution of binding sites to these ligands in mammalian brain tissue. The highest level of binding were obtained in the regions known to receive large serotonergic innervation. [H^3]-spiroperidol which binds dopamine receptors, was later shown to label a population of serotonin binding sites in the cortex. In 1979, Peroutka and Snyder supplied evidence showing that [H^3]-spiperone and [H^3]-5HT binding sites were distinct molecular entities. This led to the introduction of the notations $5HT_1$ and $5HT_2$ to distinguish these binding sites. $5HT_1$ denoted the specific binding site for [H^3]5HT while the preferential binding site for [H^3]-spiroperidol was designated $5HT_2$. [H^3]-LSD labelled both sites to a similar extent. [H^3]-Mianserin and [H^3]-ketanserin are other ligands that have been shown to preferentially label $5HT_2$ sites.

Based on inhibition characteristics of [H^3]5HT binding by antagonists, subclassification of $5HT_1$ into $5HT_{1A}$ and $5HT_{1B}$ subtypes was introduced by Pedigo and colleagues in 1981. Recently, yet another subtype, $5HT_{1C}$ (Cortes, Palacios and Pozos, 1984) and a new site, $5HT_3$ (Bradley et al., 1986) have been proposed.

Investigation of the pharmacology and physiological functions of these binding sites have been more successfully demonstrated for $5HT_2$ than $5HT_1$ sites. $5HT_2$ sites are sensitive to chronic antidepressant treatment and mediate the behavioural syndrome which follow central 5HT stimulation. In the periphery, $5HT_2$ sites have been demonstrated to mediate serotonin-impaired blood circulation, serotonin-induced

tracheal and vascular smooth muscle contraction and serotonin-induced platelet function. $5HT_1$ binding site, on the other hand, is regulated by guanide nucleotides and is associated with adenyl cyclase. There are still some controversies about the functional role of this site (Leysen, 1985). However, it is thought to mediate certain behavioural responses. For instance, evidence shows that the reciprocal forepaw treading behaviour in the rat is mediated by $5HT_{1A}$ site (Tricklebank, 1985). Some types of sexual behaviour in the rat are also believed to be mediated by this site (see section 1.1.7.c.).

1.1.7. Physiological functions of serotonin

Serotonin has been implicated in several physiological and behavioural functions both in the central nervous system and the periphery. Some of these functions are: regulation of the endocrine system, thermoregulation, sleep/wake cycle, pain perception, control of blood pressure, appetite and sexual function. In this section, available evidence is summarized to illustrate the participation of serotonin in these physiological processes. The discussion is limited to the CNS and since it is not meant to be an exhaustive review, only a few examples are chosen to illustrate the significant role of 5HT in normal physiological functions.

(a) Serotonin and the sleep-wake cycle

Detailed studies in both the cat and the rat indicate that serotonin is clearly involved in regulation of sleep (Jouvet, 1973; Rechtschaffen et al., 1973; Morgane and Stern 1973). Briefly, these studies involved examination of sleep patterns following anatomical

lesions of the raphé system and neuropharmacological interference of serotonin function by drugs, notably parachlorophenylalanine (PCPA) and chloramphetamine. As mentioned earlier, raphé nuclei are rich in serotonin-containing neurons. Its destruction was followed by a fall in serotonin levels in the brain and loss of sleep. Administration of PCPA (an inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in 5HT synthesis) also decreased serotonin levels and caused insomnia. So did administration of chloramphetamine. The loss of serotonin was proportional to the number of hours of sleep lost. Administration of 5-hydroxytryptophan (5HTP) reversed insommia in PCPA treated animals, though not in chloramphetamine treated nor raphé-lesioned animals.

Evidence suggest that there is an interaction between serotonergic and catecholaminergic systems in regulation of sleep. The locus coeruleus is mainly a noradrenergic centre and its projections to the raphé complex have been identified. Lesions in the locus coeruleus produced hypersomnia accompanied by an increased level of 5HIAA in the brain and spinal cord, suggesting an increased turnover of 5HT. α-Receptor blocking agents e.g. prazosin decrease waking and increase paradoxical sleep in rats, cats and monkeys (Monti, 1983; Leinoren and Sternberg, 1986). Selective pharmacological stimulation of pre-synaptic α_2-receptors with, e.g. clonidine decreases REM sleep while selective blockade (by say yohimbine) increases wakefulness. Similarly, chemical lesions with 6-hydroxydopamine increases slow-wave sleep. Thus though NA and DA are thought not necessary for initiation and maintenance of REM sleep, they may act to modulate it. They are however believed to be

involved in waking.

It appears therefore that a proper balance of interaction between DA, NA and 5HT is essential for normal maintenance of sleep-wake cycle. The contributory role of amino acids, neuropeptides and their interaction with monoamines may also be important, especially in view of the fact that coexistence of peptides and amino acids in the neurons involved in sleep have been described.

(b) Serotonin and feeding

Pharmacological, biochemical and behavioural studies in both humans and animals indicate that serotonin has an inhibitory influence on feeding. This belief is based on findings that direct and indirect acting agonists at central 5HT synapses reduce food intake. These drugs, apart from 5HT itself, include quipazine (5HT agonist), monoamine re-uptake blockers such as chlorimipramine and fluoxetine and agents like fenfluramine which enhance presynaptic release of 5HT.

That brain 5HT plays an important role has been confirmed by direct injection of drugs into the brain, especially the hypothalamus. Injection of 5HT into the medial hypothalamus in chronically-cannulated rats results in dose-dependent suppression of feeding. Likewise, injection of fenfluramine into the same site yield similar results. These studies and others indicate that endogenous 5HT may act by influencing termination of feeding (i.e. via induction of satiety). Other investigations have implicated the monoamine in influencing the animal's preference for specific foods. It appears to reduce ingestion of carbohydrates while sparing protein intake. Consistent with this finding is the observation that rats

injected with quipazine and fenfluramine reduce carbohydrate and fat intake. On the other hand, peripheral administration of the 5HT antagonist, cyproheptadine produces opposite effects. Similar results have been obtained in humans.

The medial hypothalamus is rich in both 5HT and α_2-adrenergic receptors and is densely innervated by both 5HT- and NA-containing neurons. It is believed that these two systems in this area interact antagonistically in control of food intake. This possibility is supported by the observation that injection of NA to this area produces opposite effects to those of 5HT. This action is blocked by 5HT and vice versa. Lesions in the paraventricular area of the medial hypothalamus induce hyperphagia, especially for carbohydrates and attenuate the anorectic action of systemic fenfluramine.

Other investigations also implicate an involvement of dopamine in regulation of feeding. In particular the lateral hypothalamus, an area rich in dopaminergic neurons and terminals appear important. Injection of dopamine or its agonists into this area reduce food intake and is antagonized by dopamine blockers e.g. chlorpromazine.

From these studies it has been suggested that serotonergic, noradrenergic and dopaminergic systems in the hypothalamus interact to regulate carbohydrate and protein intake, their time course pattern and the ratio between these macronutrients. The reader is referred to excellent recent reviews in pharmacological and behavioural aspects of serotonergic system in control of feeding (Leibowitz and Shor-Posner, 1986; Garattini et al., 1986; Blundell, 1986 and Silverstone and Goodall, 1986).

(c) Serotonin and sexual behaviour

The role of serotonin in the regulation of sexual behaviour, like most of its other functions, has been studied by observing the effects following pharmacological and anatomical interference in central serotonergic neurons in laboratory animals.

An example of measurement of sexual behaviour in male rats is noting duration and successful scores on mounting, intromission, ejaculation and refractory periods (Tucker and File, 1983). A sign of receptivity in the female when in oestrous is the lordosis posture - the back is flexed into a concave curve, tail held to one side, the neck extended and the anogenital region held high off the floor.

The medial pre-optic area of the hypothalamus, which receives ascending serotonergic fibres from the midbrain, appears to be an important centre for regulation of sexual function. Stimulation of this area by implanted electrodes increases sexual behaviour while its destruction inhibits sexual display in the male rat. However, the opposite is observed in female rats. The sexual facilitory centre in the female appears to be located in the ventromedial nucleus (Yamanouchi, 1980).

Parachlorophenylalanine has been used extensively and reports are consistent that it enhances mounting behaviour in both male and female rats, which has been interpreted as an increased male sexual behaviour. It is potentiated by pargyline, a MAO inhibitor and antagonized by 5-hydroxytryptophan (5HTP) an immediate precursor of 5HT. Similar results have been obtained with selective destruction of serotonergic neurons by intraventricular injection of neurotoxins such as 5,6- and 5,7-dihydroxytryptamine or by a 5HT depleting agent p-chloroamphetamine in both normal and castrated male rats

(Södersten et al., 1978, quoted by Tucker and File, 1983). Thus depletion of 5HT has an invigorating effect on males, especially the ones that were initially sexually sluggish.

One would predict from these observations that 5HT agonists would produce opposite effects, that is, inhibition of sexual behaviour. However, lisuride and tetralin derivatives e.g. 8-hydroxy-2-(di-n-propyl-amino)tetraline (8-OH-DPAT), both agonists at 5HT receptor sites, stimulate sexual behaviour. Similarly, 5HT receptor antagonists metergoline, methysergide and cinanserin stimulate sexual behaviour in both male and female rats.

Studies in female animals have yielded more contradictory results than those in males. In 1960s Meyerson suggested from his studies using reserpine and 5HTP that 5HT has an inhibitory effect on receptivity in females. Results obtained using PCPA have been contradictory. While some workers have found stimulatory, others have reported inhibitory or no effect on lordosis. Generally it enhances the male patterns of sexual behaviour in the female. Selective destruction of the serotonergic pathways to anterior hypothalamus, hypothalamic implantation of 5HT antagonists and administration of re-uptake blockers all had the effect of enhancing lordosis (reviewed by Tucker and File, 1983).

It has been proposed recently that these conflicting responses in both males and females are due to differential mediation by $5HT_1$ and $5HT_2$ receptors, since it is now known that the drugs or agents used may bind selectively to either of these receptors or the subtypes $5HT_{1A,B}$ or C. In females, $5HT_2$ activity facilitates lordosis while $5HT_1$ can either inhibit or enhance it. The inhibitory

effect is mediated by $5HT_{1A}$ while the facilitatory effect is by $5HT_{1B}$ (Mendelson and Gorzalka, 1986). On the other hand the male sexual behaviour is facilitated by $5HT_{1A}$ and $5HT_2$ and probably inhibited by $5HT_{1B}$ or $5HT_{1C}$ or other unidentified subtype. Generally serotonergic activity inhibits male sexual behaviour.

The catecholaminergic system probably plays an important role in this regulatory mechanism. Yohimbine, an adrenergic autoreceptor antagonist, facilitates male sexual behaviour (Clark, Smith and Davidson, 1984). This suggests that adrenergic system may be involved in this process.

In humans there are reports of moderate success in treatment of premature ejaculation by clomipramine (Goodman, 1980, quoted by Tucker and File, 1983). This would be consistent with the concept from animal studies that raised 5HT levels cause some inhibition of response. However, because of unwanted side effects, drugs acting via the serotonergic system do not appear promising for use in humans.

(d) 5HT and Aggressive Behaviour

The serotonergic system and its involvement in social interactions, irritability and aggressive behaviour has been studied extensively in laboratory animals, especially the rat. Fighting, social dominance and submission are all part of social interactions of caged rats. Aggressive behaviour has been differentiated into offensive and defensive elements, depending on underlying motivations, postures exhibited and environmental factors. Muricidal behaviour is frequently used as an index of offensive behaviour. In territorial situations, resident rats mostly display offensive while intruders largely exhibit defensive behaviour.

Depletion of serotonin in the rat brain by different mechanisms clearly facilitates various kinds of aggressive behaviour mentioned above. Systemic administration of parachlorophenylalanine or intracerebroventricular injection of neurotoxins 5,6- and 5,7-dihydroxytryptamine has consistently been reported by several investigators to increase aggressive behaviour (Vergness, Depaulis and Boehrer, 1986 and references quoted therein). A decrease in serotonergic activity has also been shown to provoke a shift from subordination to dominance (Kostowski, Plewako and Bidzinski, 1984). Systemic administration of 5HTP or intraventricular injection of 5HT reverses the aggressive behaviour induced by 5HT depletion. It has therefore been suggested that 5HT provides tonic inhibition to aggressive behaviour (Vergness, Depaulis and Boehrer, 1986). The mechanism of this process, however, remains to be elucidated.

(e) 5HT and the endocrine function

Central neurotransmitters are known to be involved in regulation of endocrine function, partly by influencing hypothalamic releasing factors which in turn control anterior pituitary function or in some cases by acting directly on pituitary or on the target organs. Administration of various monoaminergic receptor agonists or antagonists is reflected by a decreased or increased plasma levels of one or several of the pituitary hormones which can then be monitored.

The secretion of growth hormone (GH) and its influences by neuroreceptor function has been studied extensively in both humans and animals. It is secreted exclusively in episodic bursts. In the male rat the peaks occur at 3-4 hour intervals and with low or

undetectable levels between peaks (Tannenbaum and Martin, 1976). The secretion is assumed to be under the influence of somatostatin and growth hormone releasing factor (GHRF). Administration of reserpine to rats in a dose that effectively depletes DA, NA and 5HT supresses the normal pulsative secretion of GH. On the other hand, administration of PCPA in a dose that inhibits 5HT neurotransmission while leaving catecholaminergic neurons intact has no effect on secretion of GH (Eden, Bolle and Modigh, 1979). Other reports show that administration of 5HTP to reserpine-treated rats has no effect on GH secretion on its own but when given with clonidine, an α_2-receptor agonist, a dose-dependent 5HTP potentiation of GH secretion results (Balldin et al, 1980, quoted by Ericksson and Modigh, 1984). It thus appears that stimulatory influence of serotonin is probably exerted by interaction with noradrenergic system. In humans, quipazine, 5-hydroxytryptophan and l-tryptophan produce only small and variable changes in GH secretion (Charney et al., 1982). Clomipramine causes a smaller stimulation of GH than desipramine, a more selective NA uptake inhibitor (Laakman et al., 1984). From these results and others it appears that amongst monoaminergic neurotransmitters, adrenergic system exerts the strongest influence on GH secretion, followed by dopaminergic and finally the serotonergic system (Checkley, 1985).

5HT has a stimulatory role in prolactin (PRL) secretion. In animals lesions placed in the dorsal raphé nucleus is followed by an inhibitory release (Henninger, Charney and Sternberg, 1984, quoted by Checkley, 1985). In humans this effect is commonly studied using tryptophan administration. Infusion of l-tryptophan intravenously is followed by a rise in plasma PRL (Cowen et al, 1985; Anderson and

Cowen, 1986). This effect is thought to be due to increased 5HT levels in the brain and is supported by the observation that administration of clomipramine, a re-uptake blocker, potentiates the rise in plasma PRL (Anderson and Cowen, 1986). It has been suggested that the 5HT action is mediated by $5HT_1$ receptors because administration of $5HT_2$ blockers cyproheptadine or ketanserin do not have any significant effect (Cowen et al.,1985).

There is evidence that serotonin participates in adrenal-pituitary axis regulation. Administration of corticosteroids to intact animals causes an increase in tryptophan hydroxylase activity while, conversely, adrenalectomy is associated with decreased tryptophan hydroxylase activity (Krieger, 1978). The negative feedback by adrenal glucocorticoids on the pituitary is thus probably mediated through increased activity in central serotonergic neurons and the same neurons may in turn participate in ACTH regulation (Osborne, 1982).

Serotonin plays a part in gonadotropin regulation. Injection of the amine into the ventricles decrease serum levels of luteinizing hormone (Schneider and McCann, 1970). It is also known that serotonin administration produces inhibitory effect on ovulation in rodents (Labhsetwar, 1971). Futhermore, other studies indicate that serotonin inhibits release of LHRF from the mediobasal hypothalamus, though not in the anterior pituitary terminals (Charli et al, 1978, quoted by Osborne, 1982).

1.1.8. The circadian rhythm of 5HT in the CNS

The 24-hour periodicity of 5HT concentration in the brain of

rodents has been known for a long time. Among the earlier work on this subject was that by Albrecht and co-workers in 1956. They reported that in both male and female mice the highest concentration was found in the light and the lowest in the dark period. These initial findings have since been confirmed by several other investigators in mouse, rat and hamster brain (Friedman and Walker, 1968; Scheving et al., 1968; Quay, 1968; Hillier and Redfern, 1977; Okada, 1971; Hery et al., 1977; Morgan and Yndo, 1973; Ho et al., 1985). Some have determined the concentration of 5HT in discrete brain areas, for example the hypothalamus, frontal cortex, brainstem and the pineal gland. In general the rhythm is synchronized in all these brain areas. Others have attempted to correlate 5HT rhythm with that of biogenic amines, dopamine and noradrenaline, rest-activity cycle, body temperature, melatonin and brain tryptophan concentration. Generally, the rhythms of all these other variables are in phase but interestingly they are about 180^{o} out of phase with that of 5HT.

The challenge facing the interested investigator has been to elucidate how this rhythm of 5HT level is generated, which so far appears not to have been fully realized. Availability of brain tryptophan, regulation of biosynthesis and catabolism of 5HT have all been investigated as the possible regulatory sites.

Fernstrom and Wurtman (1971) demonstrated that loading rats intravenously with l-tryptophan is paralleled by a proportional rise in both brain tryptophan and 5HT. They concluded that brain 5HT levels may in part be dependent upon the availability of tryptophan. Another attempt to explain this problem is the observation that there is a diurnal variation in the initial rate of accumulation of l-

tryptophan by brain tissues. Both in vivo and in vitro studies have shown that the active transport system (see section 2.1.1.) is more efficient during the light than the dark period in the rat and it has been suggested that this may be responsible for regulating the circadian rhythm of 5HT in the brain (Hery et al., 1974). However, the fact that under normal physiological conditions the concentration of tryptophan is higher in the dark and out of phase with that of 5HT weakens these two hypotheses.

Tryptophan hydroxylase, the rate limiting enzyme in the synthetic pathway and MAO, the deaminating enzyme both exhibit circadian rhythms in their activities. The activity of tryptophan hydroxylase is highest in the dark and lowest in the light period (reviewed in Chapter 2). MAO activity on the other hand peaks during the light phase (Owasayo et al., 1984) which coincides with the highest concentration of 5HT. If these were the only regulatory mechanisms, the highest concentration of 5HT would be expected to occur in the dark period, corresponding to the higher activity of tryptophan hydroxylase and lower during the light due to higher activity of MAO. Thus the availability of l-tryptophan, the activity rhythms of the two important enzymes do not satisfactorily explain the rhythm of 5HT concentration in the brain. This problem is examined further in the discussion section.

In the rat pineal gland, however, it is believed that 5HT rhythm is controlled by that of N-acetyltransferase enzyme, to which it is inversely out of phase (Klein, 1974). This enzyme acetylates 5HT, which is the rate-limiting step in melatonin synthesis (section 1.1.4.). Its activity rises rapidly just after the onset of darkness,

followed by about 50% fall in steady state concentration of 5HT and a rise in melatonin level. In the light, its activity drops. As 5HT is no longer converted to melatonin, its concentration rises steadily to reach its peak towards the end of the light phase. The activity of N-acetyltransferase is in turn controlled by the suprachiasmatic nucleus through the sympathetic system.

Circadian systems in general and their role in health and disease (especially affective illness) is discussed further in the next section.

1.2. PHYSIOLOGY OF CIRCADIAN SYSTEMS

The earth's yearly revolution around the sun is responsible for the seasonal changes while its rotation about its axis once in about 24 hours determines the day-night patterns to which we are all exposed. Because of this highly predictable daily rhythm animals and plants have evolved over millions of years behavioural, physiological and biochemical systems that follow a 24-hour schedule.

During the course of evolution oscillations of certain frequencies in biological systems have been selected to serve as "internal clocks". They enable an organism to measure time and synchronize the organism's internal milieu with daily events in its environment. These are known as circadian timing systems. The word circadian (Latin: circa = about, diem = day) was coined by Franz Halberg in 1959 to describe the approximately 24-hour cycles that are generated by an organism. Virtually all eukaryotic organisms exhibit circadian rhythms in physiology and behaviour.

1.2.1. Examples of circadian rhythms

There are daily rhythms in almost every aspect of animal behaviour and physiology. Indeed it is difficult to demonstrate a variable which does not show such circadian variation.

The most obvious and dominant rhythm is the sleep-wake cycle. Sleep itself is composed of alternating ultradian phases, rapid eye movement (REM) and slow wave (SW) sleep. Both sleep-wake and REM-SW cycles are endogenous rhythms which persist under constant environmental conditions (Weitzman, et al., 1979; Minors and Waterhouse, 1984). The free-running period of sleep-wake cycle in humans is about 24 hours, though in some subjects studied a long time, it eventually lengthens to 30-50 hours (Czeisler et al., 1980).

Other examples of physiological systems which are subject to circadian control in animals are: feeding and drinking, thermo-regulation, endocrine functions, renal function and reproduction. These and other rhythms are described in detail in several books and reviews e.g. Moore-Ede, Sulzman and Fuller, 1982, Wever, 1979 and Conroy and Mills, 1970.

In addition to circadian systems, a wide spectrum of biological rhythms occur. The high frequency rhythms include the electrical activity in the brain (EEG), heart rate and respiratory rate. Their periods are less than 24 hours and are therefore termed ultradian rhythms. Examples of low frequency rhythms are the menstrual cycle in women and the seasonal (circannular) cycle of hibernation. Such rhythms with periods longer than 24 hours are referred to as infradian.

There are events that occur only once in a lifetime of an animal which may be of crucial importance for its survival e.g., birth

or egg hatching, metamorphosis and emergence of insects, fledglings, and in some species, copulation. There may be an optimal time of day for such events because they have been observed to follow a temporal pattern (Pittendrigh and Skopik, 1970; Daan and Aschoff, 1982).

1.2.2. Characteristics of Circadian Clocks

(a) Entrainment by environmental time cues

Internal clocks are sensitive and can only synchronize to cyclic environmental variables with periods close to 24 hours, usually 23 to 26 hours. In 1951 Aschoff coined the term Zeitgeber (from the German, literally meaning "time giver") to describe such periodic environmental variables. Other terms like "synchronizer", "entraining agent", and "time cues" are also used.

A mammal is first exposed to a cyclic environment in utero from the concentration of nutrients and hormones crossing over the placenta from maternal bloodstream. This entrains foetal circadian pacemaker to maternal circadian rhythmicity (Deguchi, 1979; Reppert and Schwartz, 1983). The newborn gradually develops overt circadian rhythms as the pacemaker entrains to environmental Zeitgebers. In newborn human infants, circadian rhythms in waking and sleeping are not initially seen, but appear after the first few months of life (Meier-Koll, et al., 1978, quoted by Moore-Ede, Sulzman and Fuller, 1982).

In the natural environment, multiple time cues exist. The light-dark (LD) cycle is an important Zeitgeber and all animals that have been studied including humans have been shown to entrain to it (Moore-Ede, Sulzman and Fuller, 1982). The changing length of

daylight throughout the year, on the other hand, is an important cue for synchronising seasonal rhythms through melatonin secretion (Arendt, 1985; Lewy, 1983).

Food availability cycles have been shown to synchronize rest-activity cycles in animals (Sulzman, Fuller and Moore-Ede, 1977a). It has recently been reported that scheduled feeling is a stronger Zeitgeber than LD cycle in entraining serum rhythms of tryptophan, serotonin and N-acetyl serotonin in the rat (HO, Chik and Brown, 1985). The role of mealtimes in humans is less convincing and is only probably a weak cue (Minors and Waterhouse, 1986).

Social contact is believed to be an important time cue in some species including mice (Halberg, Visscher and Bittner, 1954) and humans (Vernikos-Danellis and Winget, 1979) but not in others, eg the squirrel monkey (Sulzman, Fuller and Moore-Ede, 1977b, quoted by Moore-Ede, Sulzman and Fuller, 1982). In fact Wever (1979) and others have advanced the view that social contact is a stronger Zeitgeber than LD in humans. However, it is the belief of some authors that the search for the most effective Zeitgeber in humans cannot be done successfully because more than one rhythmicity may be involved and it is difficult to rule out other influences in the experimental procedure (Vernikos-Danellis and Winget, 1979; Moore-Ede, Sulzman and Fuller, 1982; Minors and Waterhouse, 1986).

Other time cues which have been demonstrated to be effective synchronizers are: sound in the hamster and the house sparrow (Menaker and Eskin, 1966) and electromagnetic field strength in mice (Dowse and Palmer, 1969) and humans (Wever, 1970, quoted by Moore-Ede, Sulzman and Fuller, 1982).

(b) Endogenous components of circadian rhythms

Circadian rhythms in an organism are endogenous in nature and persist in the absence of environmental time cues. This process is called free-running and is widespread in both the plant and animal kingdoms. Each species shows a characteristic free-running period which could be a little longer or shorter than, but rarely equal to 24 hours. Aschoff (1960) demonstrated that successive generations of mice reared in isolation from environmental time cues continued to show circadian rhythms despite the fact that none of the mice in any generation had ever seen a light-dark cycle. Richter (1968, quoted by Moore-Ede, Sulzman and Fuller, 1982) also found that a blinded squirrel monkey continued to show activity rhythms for up to 3 years.

However, not all circadian rhythms persist indefinitely in constant conditions. For example, locomotor activity rhythms in rats dampen out with time (Honma and Hiroshige, 1978). The mechanism responsible for this phenomenon is not fully understood, though it is possible that several clocks involved in the system uncouple in constant conditions and free-run independently (Moore-Ede, Sulzman and Fuller, 1982).

The period of a circadian clock is independent of temperature changes. The rate of most biochemical processes changes two- to three-fold with a 10^{o}C change in temperature. Evolution has therefore created mechanisms to compensate for changes in temperature and thus ensure uniformity of cicardian clocks. However under extreme conditions (0^{o}-5^{o}C) circadian clocks can shut down altogether (Richter, 1975 quoted by Moore-Ede, Sulzman and Fuller, 1982; Vanecek, et al., 1985).

The intensity of light can alter the length of a free-running

period of a circadian clock in a constant environment. These changes are usually opposite for diurnal and nocturnal animals. This observation first noted by Aschoff (1960), led to the famous Aschoff's Rule: as light intensity increases, the rhythm period decreases in diurnal animals, while under the same conditions, the period increases in nocturnal animals. Aschoff's Rule has been supported by studies in several animal species though exceptions have been reported in both human (Wever, 1979) and non-human primate species (Fuller and Edgar, 1986) where the periods have been found to be shorter or longer than predicted.

(c) Genetic basis of the circadian pacemakers

Genetic studies of circadian systems especially in lower animals have revealed that circadian clocks are genetically programmed and inherited by successive offspring. For example Drosophila wild type strain has a free-running period of 24.2 hours. Mutants with 19- or 28-hour periods and arrhythmic mutants have been derived. Each mutant has been mapped to the same genetic locus on the X-chromosome, suggesting that specific "clock genes" do exist (Konopka and Benzer, 1971). Proteins and biological structures synthetized by clock genes have been identified (Konopka and Wells, 1980, quoted by Moore-Ede et al.,1982). Hall (1986) has recently reviewed behavioural changes (especially those involving sexual behaviour) as a result of mutations of the circadian clock in the Drosophila. A new scope has therefore been opened in the study of the biology of the circadian clocks. It is feasible to envisage studies using, for example, recombinant DNA techniques, that may eventually unravel the mysteries

of the mammalian clocks.

1.2.3. Benefits of circadian timing systems

The circadian timing system performs a very important function in ensuring that behavioural and internal metabolic functions of an organism are appropriately timed with respect to periodic events in the environment. Most species adopt a characteristic temporal pattern suitable to their ecological niche. Thus some animals are nocturnal or diurnal while others may confine their activity mostly to a specific time of day e.g. dawn or dusk (crepuscular). This evolutionary process allows for avoidance of not only competition for food supplies but also predators whose behaviour may also be cyclic. This ensures maximal survival of the species in a difficult world. The reader is referred for further details on the significance of biological rhythms to a review by Daan and Aschoff (1982).

1.2.4. Identification of the pacemaker

The hypothalamus was identified by Richter in 1965 after several decades of study as the location of the biological clock. Using free-running activity rhythm of blinded rats as a marker, he subjected the animals, in his own words, to "almost every conceivable kind of metabolic, endocrinologic and neurologic interference... to no avail" (Richter, 1965 quoted by Moore-Ede, Sulzman and Fuller, 1982). This included removal of adrenals, gonads, pituitary, thyroid, pineal or pancreas, electroshock therapy, induced convulsions, prolonged anaesthesia and alcoholic stupor. After placing hundreds of lesions in the rat brain, he discovered that only those in the hypothalamus resulted in the loss of circadian

rhythmicity in activity, feeding and drinking behaviour. The precise location in the hypothalamus remained unknown until 1972 when it was reported that lesions which destroyed the suprachiasmatic nucleus (SCN) also abolished rhythms in drinking and locomotor activity (Stephan and Zucker, 1972). This has been confirmed since by several other investigators that the SCN is indeed integrally involved in generation of circadian rhythms (Rusak and Zucker, 1979; Moore, 1982, 1983; Nagai, Mori and Nakagawa, 1983 quoted by Minors and Waterhouse, 1986; Rietveld, 1985).

A different line of investigation suggests, however, that the SCN is not the only location of the pacemaker. Lesions of the SCN in the squirrel monkey leads to a loss of the circadian drinking rhythm but not the circadian body temperature rhythm (Fuller et al, 1981). Secondly, studies in humans in isolation have shown that different rhythms desynchronize and free-run in two groups, one group coupled to the sleep-wake cycle while the other follow the core body temperature (Wever, 1979; Aschoff, 1965, Czeisler, et al., 1980). The oscillator in the SCN is believed to be influenced by the light-dark cycle (Kronauer, et al., 1982) while temperature influences the second pacemaker, the location of which is unknown. The ventromedial nucleus of the hypothalamus (VMH) or the lateral hypothalamus have been suggested as the possible sites (Moore, 1982,1983; Inouye, 1983 quoted by Minors and Waterhouse, 1986). There is ample evidence that the two oscillators are mutually coupled. This ensures normal internal synchronization of the circadian system (Czeisler, et al, 1980; Zulley, Wever and Aschoff, 1981, quoted by Minors and Waterhouse, 1986).

It may be worth pointing out here that the evidence for a second pacemaker in humans has been obtained indirectly from studies on the different forms of internal desynchronization (Folkard, Minors and Waterhouse, 1985). This is because for ethical reasons ablation of the SCN cannot be performed.

Moore-Ede and his colleagues have proposed the presence of "secondary" or "peripheral" oscillators to explain overt rhythmicity observed in isolated tissues and organs, e.g. the adrenal steroids in adrenal glands (Moore-Ede, Sulzman and Fuller, 1982; Moore-Ede, 1983). They postulate that these oscillators are not autonomous but are driven by the pacemakers. Their rhythms dampen and eventually disappear in the absence of the driving pacemaker input.

1.2.5. Neurochemistry of the suprachiasmatic nucleus

The biochemical events in the SCN associated with the generation of circadian rhythms are not fully understood. Studies have identified a number of neurotransmitters and peptides within the SCN and these include: serotonin, acetylcholine, vasopressin, somatostatin, corticotropin releasing factor, vasoactive intestinal peptide (VIP) and avian pancreatic polypeptide (APP). The distribution of these neurotransmitters and peptides in the SCN is specific and this probably relates to their functional role.

Administration of carbachol, a cholinergic agonist, to laboratory animals mimics the effects of light in as far as phase-shifting and entraining of various circadian rhythms are concerned. This has raised the speculation that acetylcholine may be involved in relaying light information within the SCN (Turek, 1985). On the other hand, administration of APP mimics the effects of darkness.

Iontophoretic administration of serotonin or stimulation of dorsal raphe nuclei, a major serotonergic projection to the SCN, both suppress the firing of SCN cells (Groos, Mason and Meijer 1983). This suggests that serotonergic activity has an inhibitory effect on the pacemaker. In contrast, administration of acetylcholine increases the firing rate of SCN neurons.

Recent studies using in vivo voltammetry have demonstrated that 5HT turnover and release are highest at the onset of darkness in the SCN of rats (Martin and Marsden, 1985). It is interesting that this period also corresponds to the highest activity period in activity-rest cycle in rodents. Though this would indicate that the serotonergic neuronal activity in the SCN is highest in the dark, there are conflicting reports from electrophysiological studies showing that the firing rate of SCN neurons is highest in the light (Inouye and Kawamura, 1979).

1.2.6. Circadian rhythms in health and disease

(a) Consequences of circadian phase shift

A rapid environmental phase shift in the form of a flight across multiple time zones or a change in shift-work introduces a stress which manifests itself in general symptoms including disruption of sleep, gastrointestinal disturbances, deterioration in performance and a general feeling of malaise. The severity of the symptoms varies with individuals and may last several days as the body's circadian system synchronizes to the new environmental time cues. If it is caused by a flight, it is termed "jet lag" and can be minimized by maximum exposure to the new environmental Zeitgebers, especially

social cues (Klein and Wegmann, 1974). The direction of the flight also influences the rate of resynchronization. A westward flight phase-delays the circadian system and is followed by a more rapid readjustment than an eastward one (phase-advancer) (Klein and Wegmann, 1979, quoted by Moore-Ede, et al, 1982). While the symptoms of "jet lag" may be transient, exposure to several years of shiftwork may induce serious sleep disorders, gastrointestinal tract and cardiovascular diseases (Moore-Ede and Richardson, 1985). That some agents e.g. theophylline, pentobarbital, short-acting benzodiazepines and dexamethasone have been demonstrated to possess phase-shifting (i.e. "clock-resetting") properties has raised the possibility of developing a jet lag "pill" (Ehret, Potter and Dobra, 1975; Horseman, Meinert and Ehret, 1979; Seidel et al., 1984).

(b) Circadian rhythms in diagnosis and treatment of disease

As discussed earlier, almost every physiological and metabolic system displays a circadian rhythm. It is important therefore that time of day be taken into account when measuring any physiological variable which may be of diagnostic value. For example, plasma cortisol concentration, which has been used as a marker in diagnosis of various diseases e.g. Cushing's Syndrome, Addison's disease and endogenous depression, is higher in the morning than in the evening.

Effectiveness and toxicity of many drugs have been shown to follow a circadian rhythm (Halberg, 1969; Reinberg and Halberg, 1971; Moore-Ede, 1973). The efficacy of a drug is a function, in part, of the rate of absorption, metabolism and excretion, the degree of dilution in body fluids and susceptibility of the target tissue. Circadian rhythmicity in all these factors have been demonstrated and

thus all contribute to the overt rhythm of drug effectiveness. The usefulness of this knowledge is being increasingly recognised in clinical cases where the use of drugs with low safety margins may be necessary, e.g. in cancer chemotherapy. In this condition, the tumour as well as the host cells have a rhythm of resistance and susceptibility. It has been demonstrated that success of treatment is improved significantly when circadian factors are taken into account (Moore-Ede, Sulzman and Fuller, 1982; Hrushesky, 1985).

1.2.7. Circadian rhythms in affective illness

The circadian system in a healthy organism is highly organised in a stable temporal order. Disorders in the system have been observed in some psychiatric diseases and this has raised speculations that the circadian system may be involved in the aetiology of at least some psychiatric illnesses.

A number of hypotheses have been proposed to account for the role of the circadian system in affective disorders, among which are: 1) The desynchronization hypothesis (Halberg, 1968, quoted by Zerssen et al., 1985) according to which the circadian rhythm of some physiological variable is no longer entrained to 24-hour schedule of the environment and other circadian rhythms, probably due to an impairment of the circadian pacemaker. 2) The phase-advance hypothesis (Papousek, 1975, quoted by Wehr et al., 1983) which assumes an early timing of physiological functions relative to the socially determined 24-hour schedule. This could be due to a general impairment of internal coupling mechanisms or dysfunction of the "clock" itself.

The phase-advance hypothesis has received a lot of support from several investigators and is reviewed here only briefly. It has long been recognised, for example, that depressed patients wake up early and are unable to fall back to sleep (Mendels and Cochrane, 1968). Another common symptom is the diurnal variation in mood - the condition is worse in the morning but improves by the evening. Statistics amongst the general population show that affective disorder (at least the bipolar type) is characterised by regularly recurring winter depressions, often alternating with summer hypomania (Rosenthal, Sack and Wehr, 1983).

Wehr et al,(1983), Wehr and Goodwin (1983) and Kripke (1983) have reviewed circadian rhythms of several physiological parameters in affective disorders. In general, when depressed patients are compared with controls, the rhythms are either blunted (reduced amplitude) or phase-advanced. For example, the distribution of REM sleep is normally skewed, with an increasing amount occurring in the second half of night's sleep. In depressed patients the situation is reversed, with more REM occurring in the first half of the night. When bipolar depressives switch over from depressive to manic episode, they experience an abnormal 48-hour sleep-wake cycle, probably due to intrinsic slowing of the driving pacemaker.

Other rhythms phase advanced in affective illness include: temperature, urinary 3-methoxy-4-hydroxyphenylglycol (MHPG, the main metabolite of NA), activity rhythms and urinary electrolyte. The rhythms of cortisol, prolactin and melatonin are mainly phase-advanced and blunted as well (see also section 1.3.2.). In some depressives, the rhythm of melatonin is phase-delayed while phase-advanced in others. In the light of this finding it has been

suggested that there may be two subtypes of depressives: those with phase-advanced and phase-delayed circadian rhythms (Nair, 1984; Lewy, 1984, quoted by Nair, 1984).

The effect of antidepressant medication on circadian rhythms supports the phase-advance hypothesis of depressive illness. Lithium, which is used mainly as a prophylactic agent, slows free-running wheel activity rhythms in blinded rats (Kripke and Wyborney, 1980). In humans it has been shown to lengthen periods of rectal temperature, sleep-wakefulness and activity rhythms under free-running conditions (Johnsson et al, 1982). It has also been demonstrated to shift the phase relationship between temperature and sleep-wake rhythms in humans (Engelman et al, 1983).

Largely similar results have been reported with pharmacological manipulation with other antidepressants. Chronic administration of clorgyline, a monoamine oxidase inhibitor, lengthened the rest-activity cycle in hamsters. Chronic clorgyline and imipramine also induced dissociation of activity rhythms in the same animals (Wirz-Justice and Campbell, 1982).

Manipulation of sleep-wake cycle has been shown to have antidepressant effects. For example, total or partial deprivation of sleep in the second half of the night induced transient remission of depression. Interestingly, deprivation of sleep in the first half of the night had no antidepressant effect. Advancing the time of sleep by about 6 hours also induced remissions lasting about one week (Wehr et al, 1983).

Patients treated with combinations of antidepressant drugs and sleep deprivation have been reported to improve more rapidly than

those treated with antidepressants alone. Similarly, advancing the time of sleep in combination with antidepressant drugs had a potentiating effect (Sack et al, 1985). Patients with seasonal affective disorders have also been successfully treated by exposure to bright light for brief periods in the evening (Rosenthal et al, 1985; James et al, 1985).

These and other observations led to the hypothesis that lithium, monoamine oxidase inhibitors and tricyclic antidepressants all alleviate depression by slowing or delaying abnormally phase advanced circadian rhythms (Wehr and Wirz-Justice, 1982; Wehr and Goodwin, 1983).

1.3. THE BIOLOGY OF AFFECTIVE DISORDER

1.3.1. Introduction

Depression and mania, being disorders that affect mood, are categorised as affective illnesses. Both syndromes not only affect mood but many vegetative functions as well. During a depressive episode, the patient's level of energy and physical activity is often reduced. Thought processes, concentration and memory is impaired. The patient feels sad, hopeless and pessimistic about the future. Sometimes this state of mental anguish and hopelessness leads to suicide. Physical changes associated with depression include: disturbed sleep, weight loss, decreased appetite, constipation and decreased libido. The severity of depression, as cited earlier, typically shows a diurnal variation, worse in the morning but better by the evening. Often it exhibits a seasonal pattern amongst the whole population, the incidence being higher in the late spring (Rosenthal, Sack and Wehr, 1983).

Patients who experience only depression are termed "unipolar" to distinguish them from "bipolar" patients, who are also subject to manic episodes. Mania, in constrast to depression, is associated with increased energy, physical activity, libido, reduced need for sleep and the patient seeks social contact. Thinking is rapid and may be generally unrealistic. Bipolar patients, unlike unipolar ones, may be characterised by increased sleep and weight gain.

Affective illness is more common in women than men. Its cause is unknown. Predisposition is partly genetic, the genetic factor being estimated to contribute 50-60% of the cases. Incidence in first degree relatives of patients is 15-20%. The onset of bipolar illness is typically in the second and third decade of life while the onset in unipolar patients is often later.

Depressive illness has often been classified into endogenous and reactive depression. Endogenous depression occurs without any obvious causes. Reactive depression, on the other hand, is a milder form precipitated by external events. Untreated, depressive episodes may last between 6 and 18 months and the patients may improve spontaneously. The course of the disease is often marked with relapses and remissions.

1.3.2. Physiological and biochemical abnormalities in depression

Depressive illness is associated with physiological abnormalities in mainly three areas: endocrine, electrolyte and neurotransmitter function. These are currently subjects of intense investigation in different parts of the world. They are only reviewed here briefly.

(a) Electrolyte disturbance

Marked changes have been demonstrated in the transport of sodium into the cerebrospinal fluid (CSF) in depression. Raised "residual" (mainly intracellular) levels have been observed in depressives (Coppen 1960, 1965; Colt et al., 1982 quoted by Coppen and Wood, 1985). If these whole-body changes in residual sodium also occur in the CNS, it it possible that it may have profound effects in neuronal functioning. For example, the decreased activity of Na^+- K^+ ATPase observed in depressives may be responsible for increased residual sodium and decreased 5HT uptake (Hesketh, Glen and Reading, 1977).

Hypo- and hyperparathyroidism is associated with some severe affective cases. This is certain to influence the levels of calcium, which is known to have a profound effect on the CNS. However, the link with depression and mania is not fully understood (Coppen and Wood, 1985).

(b) Endocrine disturbance

The interest in possible endocrine abnormality in affective illness was spurred by the observation that in some well defined cases it was due to over-activity or under-activity of the adrenal cortex (Coppen, 1967, Brooksbank and Coppen, 1967).

The "dexamethasone suppression test" (DST) reveals a marked neuroendocrine disorder in about 70% of depressive cases (Carrol, 1982; Coppen et al, 1983). The adrenal cortex secretes cortisol under the influence of the pituitary adrenal corticotrophic hormone (ACTH), which is in turn secreted in response to hypothalamic hormone corticotrophin releasing factor (CRF). Cortisol secretion exhibits a

circadian rhythm. Secretion reaches a peak at the end of the night and then declines during the day and ceases altogether just before midnight. The balance of cortisol secretion is maintained mainly by levels of cortisol which exerts a strong negative feedback upon both ACTH and CRF secretion. The synthetic steroid dexamethasone acts through this same negative feedback mechanism. Thus a normal subject given 1-2 mg of dexamethasone at 11.00 pm secretes virtually no cortisol the next day. The "dexamethasone resistance" phenomenon occurs when this feedback mechanism fails. There are several reports of abnormalities of DST in depressives and that it returns to normal on recovery. This phenomenon has been reviewed recently by Checkley (1985) and Braddock (1986).

Another well established cause of depressive illness is myxoedema which can be treated satisfactorily if diagnosed early (Checkley, 1982 quoted by Coppen and Wood, 1985). Decreased levels of tri-iodothyronine, which also indicates thyroid under-activity, has been reported in depression (Joffe et al., 1985). The action of antidepressants has been shown to be enhanced by concomittant administration of thyroxine (T_3) (Goodwin et al, 1982). Small doses of T_3 have been successfully used to manage patients who failed to respond to lithium or other antidepressants (Coppen and Wood 1985). All these observations point to a clear, yet undefined, link between thyroid function and depressive illness. This effect is probably mediated through central α_1-receptor sites since it has been shown that thyroid hormone administration increases both monoamine synthesis and α_1-responsiveness in the rat (Svensson, 1984) or possibly through the serotonergic system since it has been

demonstrated that thyroid function has an influence on 5HT-mediated responses in laboratory animals which can be potentiated by antidepressants (Atterwell, 1981; Brochet et al, 1985).

Dopaminergic, noradrenergic and possibly serotonergic systems are involved in the secretion of growth hormone (GH) from the pituitary. Dopaminergic agents e.g. amphetamine, which releases DA or apomorphine (DA agonist) administered to normal subjects stimulate GH release. This response is reduced in depressives. Similarly the normal stimulant effect on GH release by clonidine, α_2-agonist, is reduced in depressed patients. After treatment the response to these agents returns to normal (reviewed by Matussek, 1984 and Checkley, 1985).

The secretion of melatonin and prolactin is also abnormal in depression. Melatonin secretion exhibits a circadian rhythm under the influence of the suprachiasmatic nucleus. This effect is mediated by β_1-receptors (see also section 1.1.4.). In normal subjects, melatonin is secreted at night and is inhibited by light. Several investigators have reported a reduced nocturnal secretion of melatonin in depression (Checkley, 1985). A phase advance in its rhythm has also been reported in depressives (Lewy, 1984, quoted by Checkley, 1985). After treatment of these patients with desipramine melatonin secretion returns to normal (Thompson et al, 1985, Checkley et al., 1985).

Normally, prolactin (PRL) secretion is highest at night. Nocturnal secretion appears to be abnormal in depression, probably because its secretion is linked to sleep which is abnormal in depression. Administration of l-tryptophan stimulates release of PRL (see also section 1.1.7.e.). In depression the response is

diminished and this suggests a defect in serotonergic control (Checkley, 1985).

(c) Abnormality of neurotransmitter function

The main impetus for the study of monoamines in depression came from the development of antidepressants. Imipramine was first introduced in 1957. Many other antidepressants have been marketed since. Though their pharmacology has been investigated extensively, their mode of action is still largely unknown. Nevertheless, the research has contributed significantly in understanding the biochemistry of neurotransmitter function. This has led over the years to postulation of biochemical theories of depression, the main of which are i) amine deficiency hypothesis, ii) post-synaptic monoaminergic receptor supersensitivity and iii) cholinergic hypersensitivity.

(i) Monoamine deficiency hypothesis

Monoamine deficiency hypothesis is by far the most widely quoted. It states that depression is caused by an absolute or relative deficiency of the two monoamines, 5HT (Coppen, 1967) and noradrenaline (Schildkraut, 1965) available for neurotransmission at the receptor sites in the central nervous system. The evidence which gave rise to this hypothesis was the observation that substances which depleted central monoamines such as reserpine could precipitate depressive illness while drugs that increased central monoamine levels, such as monoamine oxidase inhibitors, tricyclic antidepressants, tryptophan and 5HTP were also effective as

antidepressants.

Studies on concentration of monoamines and their metabolites in the CSF or post-mortem brains of suicides also lent support. In 1966 Ashcroft and colleagues reported that concentration of 5HIAA is reduced in CSF and post-mortem brains of depressives (reviewed by Coppen and Wood, 1982). Furthermore, the rate of accumulation of 5HIAA in CSF following probenecid administration (hence rate of 5HT synthesis) is reduced in depressives. Similarly, concentration of 3-hydroxy-4-methoxyphenylglycol (MHPG), the main metabolite of NA, is reduced in CSF and post-mortem brains of depressives (reviewed by Van Praag, 1982).

It might be worthwhile taking note here of the fact that interpretation of the data on concentration of metabolites has been criticised on several grounds. For example, the origin of 5HIAA in the CSF may not be wholly central and that suicide cases may not all be depressives (Green and Constain, 1981).

(ii) Postsynaptic receptor supersensitivity

The biogenic amine deficiency hypothesis has been severely challenged by the discovery of clinically effective antidepressants that do not inhibit either monoamine oxidase (MAO) enzyme or the uptake of biogenic amines into presynaptic nerve endings. Furthermore clinically, therapeutic response to antidepressant treatment occurs only after long-term administration, in constrast to re-uptake blockade or MAO inhibition, both of which are observed in a matter of minutes. In addition, some agents are known to increase the concentration of monoamines in the CNS e.g. cocaine (a moderately potent NA re-uptake blocker at the nerve endings) yet they are

ineffective as antidepressants. An alternative explanation was therefore necessary.

Studies on delayed post-synaptic receptor mediated events soon yielded new discoveries. In the late 1970s, it was discovered that chronic but not acute treatment with antidepressants, including electroconvulsive therapy (ECT), reduced the sensitivity of noradrenaline receptor-coupled adenylate cyclase system in the brain (Ventulani and Sulser, 1975; Ventulani et al, 1976) and that this was accompanied by a decrease in the density of β-adrenoceptors as determined by ligand binding studies with tritiated dihydroalprenolol (Banerjee et al., 1977). These initial findings have since been confirmed in many other laboratories (reviewed by Sulser, 1984). It has therefore been suggested that central adrenergic receptors are supersensitive in depressive illness and that antidepressants act by down-regulating the sensitivity of adrenergic receptors. This hypothesis has been extended to include the serotonergic systems as well since it has been shown that antidepressant treatment down-regulate central 5HT receptors (reviewed by Ogren and Fuxe 1985).

(iii) Cholinergic system

Investigations reveal that cholinergic system is involved in pathogenesis of affective disorders. Though the nature of the defect is unknown, it is generally believed that muscarinic postsynaptic supersensitivity is a major factor.

Evidence implicating the cholinergic system includes: 1) drugs activating cholinergic system produce depressive symptoms and

neuroendocrine and polysomnographic data typical of endogenous disorders; 2) drugs that directly block postsynatpic central muscarinic receptors have antidepressant effect and produce euphoria. They also up-regulate and supersensitize cholinergic system; 3) most antidepressant drugs also have anticholinergic properties.

Administration of cholinergic agents to laboratory animals produce an increased activity of noradrenergic system as manifested by increased release and synthesis of noradrenaline both in the brain and in the periphery. Hyperactivity in animals following administration of carbachol, a cholinergic agonist, into the limbic system is believed to be mediated by the monoaminergic system.

Evidence therefore points to a balanced interaction between cholinergic and monoaminergic system and that hypercholinergic system leads to depression while a hypermonoaminergic system results in mania (reviewed by Dilsaver and Greden, 1984; Dilsaver, 1986).

Chapter 2

2. CIRCADIAN RHYTHM OF TRYPTOPHAN HYDROXYLASE IN THE RAT BRAIN

2.1. REGULATION OF 5HT SYNTHESIS

The synthetic pathway of 5HT from L-tryptophan (TRY) is outlined in section 1.1.4. Evidence is reviewed here to highlight the significance of the availability of L-tryptophan, the other co-substrates and the intrinsic activity of tryptophan hydroxylase (Try-OHase) in the synthesis of 5HT. The roles of the spontaneous firing rate of serotonergic neurons and the pre-synaptic 5HT autoreceptors are discussed in Chapter 5.

2.1.1. Tryptophan availability in the brain

TRY is unique among other amino acids in that it is extensively bound non-covalently to serum albumin. Under normal physiological conditions only about 10% of TRY is freely diffusible and is in rapid equilibrium with bound TRY. This binding is specific for L-TRY. The D-isomer is not bound. Only the free, unbound TRY can cross the blood-brain barrier and enter the brain tissue.

Curzon, Wurtman and their colleagues have demonstrated independently in laboratory animals that brain concentration of TRY can be varied by manipulating plasma TRY through diet or peripheral injection of TRY (reviewed by Curzon, 1985). Depriving rats of food for 24 hours, feeding them with TRY-rich diet, or TRY-free but high carbohydrate diet all increase brain TRY and 5HT synthesis. They explained the effect of high carbohydrate diet in terms of insulin which increases uptake by muscle and hence decreasing the plasma concentration of large neutral amino acids (mainly phenylalanine, tyrosine, leucine, isoleucine and valine). The concentration of plasma TRY is largely unaffected. Since these amino acids compete

for the same uptake system, their effective removal from plasma increases the transport of TRY into the brain. Deprivation of food, on the other hand, results in mobilization of body fat and an increase in free fatty acids (FFA) in plasma which also binds to albumin, thus displacing TRY. The result is an increase in the amount of free TRY available for transport into the brain. Though FFA binds to a different site, there seems to be some overlap or an interaction with the TRY-binding site (McMenamy, 1965). Long-term deprivation of TRY, however, eventually decreases both plasma and brain TRY and hence, brain 5HT synthesis.

Parenteral introduction of a TRY load to rats (Fernstrom and Wurtman, 1971) mice (Carlsson et al., 1972) and dogs (Moir, 1974) has been shown to lead in a few minutes to a rise in TRY and 5HT concentration in brain tissues. Similarly, infusion or an oral dose of TRY induced an increase of 5HT in human frontal cortex (Curzon, 1981) and CSF (Young and Gauthier, 1981) respectively.

Any pharmacological or environmental treatment that affects plasma TRY binding can also effect brain 5HT. Drugs such as salicylates, indomethacin and phenothiazines displace TRY from albumin binding and thereby raise the concentration of free TRY. Similar results are obtained when animals are subjected to stressful situations, for example, electric shocks or simply removing rats from a communal cage and keeping them singly. This has been attributed to stress-related lipolysis, leading to increased FFA in the plasma which displace TRY albumin binding (Curzon, 1981).

Curzon and co-workers have proposed the use of the ratio of free to total plasma TRY as an index of availability of TRY uptake into

the brain while Wurtman and his group have suggested that competing amino acids should be taken into account (Curzon, 1985).

The passage of TRY from plasma through the blood-brain barrier into cerebrospinal fluid (CSF) and uptake at the neuronal or glial cell membranes involve carrier-mediated as well as non-saturable diffusion process (Pratt, 1979; Sourkes, 1979). This has been demonstrated in experiments with brain slices or synaptosomal preparations. The presence of high and low affinity uptake mechanisms and the observations that newly arrived TRY is preferentially converted to 5HT has lead to the suggestion that TRY in the brain is probably distributed in two compartments (Hamon et al 1974). The high capacity uptake system which under normal circumstances accounts for about 60% of TRY influx into the brain is subject to competition by the large neutral amino acid while the low capacity system is not (Mans et al, 1979). Any alteration in the efficiency of the carrier system would therefore be expected to have an effect on the transport of TRY and consequently of brain 5HT.

Plasma and brain TRY concentration normally show circadian rhythms. In rodents the plasma concentration of free and total TRY (and the ratio free/total TRY) peaks during the dark and this is easily attributable to the fact that these animals consume most of their food during this period (Morgan and Yndo, 1973). Thus plasma TRY rhythm is influenced by food intake. The rhythm of brain TRY is in turn dependent upon free plasma TRY (Hery et al., 1977; Morgan et al., 1975). In general, brain TRY and 5HIAA are higher in the dark than light period and out of phase with that of brain 5HT (Hery et al, 1977).

2.1.2. The role of tryptophan hydroxylase (Try-OHase)

(a) Historical note and distribution of TRY-OHase

Gal and co-workers (1963) reported the first evidence of tryptophan hydroxylase in the brain. They found that intracerebral administration of radioactive TRY to the rat and the pigeon led to formation of labelled 5HT. Soon after, Graham-Smith (1964, 1967) demonstrated the presence of a specific tryptophan hydroxylase in brain tissues of rabbits and dogs. Since these initial discoveries were made it has been confirmed by several other investigators that TRY-OHase is indeed present in the mammalian brain and that it is not uniformly distributed. The pineal gland has the highest activity. The brainstem, hypothalamus, caudate nucleus and amygdala have relatively higher activity than the rest of the brain. Activity is also present in the rat pituitary, lumbosacral region of the spinal cord and the nerve endings (Renson, 1973; Green and Graham-Smith, 1975).

(b) Regulatory properties of tryptophan hydroxylase

Try-OHase (or EC 1.14.16.4, L-tryptophan; tetrahydropteridine: oxygen oxidoreductase) is a mixed-function oxidase. It requires three substrates: L-try, molecular oxygen and a reduced pteridine cofactor, now considered to be L-erythro-tetrahydrobiopterin (2-amino-4-hydroxy-6[L-erythro-1'2'-dihydroxypropyl]-5,6,7,8-tetrahydropteridine). In the hydroxylation reaction, one atom of molecular oxygen is used in the 5-hydroxylation of TRY while the other is reduced to water. Electrons are donated by the tetrahydrobiopterin (BH_4) cofactor and the unstable quinonoid dihydropterin that results is immediately converted back to the BH_4

form by NADH (NADPH)-linked dihydropteridine reductase. For every mole of BH_4 oxidised one mole of 5-hydroxytryptophan is generated (Kaufman, 1974). The whole reaction can be represented as follows:

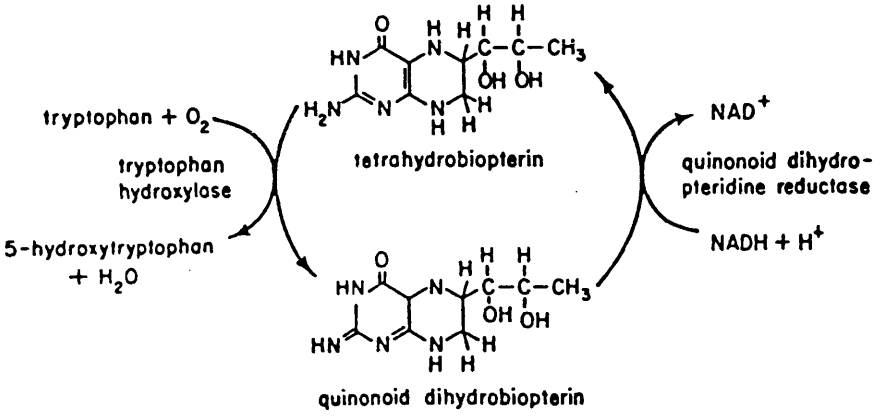


Fig.2.1 The regenaration of tetrahydrobiopterin

It is evident from this schematic outline that the activity of Try-OHase can be limited by the availability of TRY, oxygen and BH_4. The contribution of each of these substrates is now discussed in more detail.

The role of TRY has been dealt with in the last section. Suffice it to say here that under normal physiological conditions the Km of TRY-OHase for TRY in rats and rabbits (20-50 μM) is above the tissue concentration of TRY in the brain, which is about 30 μM (Carlsson and Lindqvist, 1978a) assuming a uniform distribution, though there is evidence to the contrary (Neckers et al., 1977). The enzyme therefore operates below capacity and any increase in TRY subsequently leads to a higher rate of 5HT synthesis. However, Lovenberg

and Kuhn (1982) have argued that availability of TRY *per se* may not be that critical because of the observations, among others, that a nine-fold increase in TRY concentration is followed by only about 1.5 fold increase in 5HT and that 5HT level reaches maximal value 60 min after a load of TRY which takes only about 10 min to reach its maximum concentration. Nevertheless, it is known that under certain conditions of severe depletion of TRY in the brain there is an activation of TRY-OHase. Its Vmax for TRY is enhanced by about 15-50%. This is believed to help in maintaining 5HT synthesis within normal range, thereby compensating for inadequate amounts of TRY (Boadle-Biber, 1982).

Though precise values of oxygen tension in serotonergic neurons in brain tissues have not been measured, estimates place it within the range of the Km of Try-OHase for O_2, which is about 2.5% in the rabbit (Friedman, Kappelman and Kaufman, 1972). It has also been demonstrated (as would be predictable) that an increase in the partial pressure of O_2 in inspired air is accompanied by a proportional rise in the rate of synthesis of 5HT in the brain. Similarly, a decrease in oxygen tension diminishes the activity of TRY-OHase (Davis et al, 1973; Davis and Carlsson, 1973).

Another possible regulatory centre for the activity of TRY-OHase is at the tetrahydrobiopterin (BH_4) site. This cofactor is synthesised in the brain from guanine triphosphate (GTP). Its concentration in the rat brain ranges from 0.5 to 1.0 μM and its distribution correlates with that of the activities of Try-OHase and tyrosine hydroxylase (Levine, Kuhn and Lovenberg, 1979). Since its concentration is below the Km of Try-OHase, it is likely that its availability may be limiting on the activity of the enzyme. However,

this possibility has not been successfully tested directly because addition of the cofactor to *in vitro* systems e.g. synaptosomal preparations or its injection intraventricularly prior to isolation of synaptosomes have drawn a blank (Bullard et al, 1978). On this basis it has been argued that saturating levels of BH_4 may not be important, but rather the rate of its regeneration from quinonoid dihydrobiopterin (QDHB) (Gal, 1981). As explained, earlier this process is catalysed by NADH (or NADPH)-dependent QDHB reductase. The activity of this enzyme or the availability of NADH (or NADPH) could also exert a regulatory role on TRY-OHase.

Synthetic forms of the pteridine cofactor notably 6-MPH_4 (2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydrobiopteridine) and $DMPH_4$ (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine) have been used extensively in the studies of Try-OHase activity. They have the advantage of being more stable than the natural BH_4. With the use of these forms of the cofactor Km for O_2 and Try are much higher than those obtained with BH_4 and this led in the past to erroneous interpretations concerning the role of the concentrations of these substrates (Kaufman, 1974). Secondly, it has also been reported that high concentrations of L-try inhibit Try-OHase *in vitro* in presence of BH_4 but not the synthetic forms of the cofactor (Friedman, Kappelman and Kaufman, 1972).

Although Try-OHase is not normally inhibited by moderately high concentrations of 5HT *in vitro*, there is evidence that an *in vivo* increase in the amount of 5HT brought about by say, MAOI treatment, can inhibit its own synthesis, though this was only observed in forebrain and not in the midbrain raphe (Tappaz and Pujol, 1980).

For this reason, it has been suggested that analogous mechanisms may have a part to play in the physiological regulation of 5HT synthesis (Boadle-Biber, 1982).

Besides the availability of TRY, O_2 and BH_4, regulation of 5HT synthesis could also involve an alteration in the intrinsic activity of TRY-OHase (Hamon et al, 1981a,b). For instance, it has been proposed that one mechanism for activation of the intrinsic activity of this enzyme is induced by Ca^{2+}-dependent protein kinase which phosphorylates it leading to a loss of a regulatory subunit (Hamon et al., 1981a).

2.2. EVIDENCE FOR CIRCADIAN RHYTHM OF TRYPTOPHAN HYDROXYLASE ACTIVITY IN THE CNS

The study of the circadian rhythm of Try-OHase has attracted wide interest amongst investigators mainly because it catalyses the rate limiting step in the synthesis of 5-HT. It was therefore the obvious choice in the attempt to account for the circadian rhythm in the concentration of 5HT so well documented in brains of laboratory animals.

Controversy has persisted on whether or not the activity of Try-OHase is subject to circadian rhythm. Whilst some workers have reported the presence of the rhythm, others have not. Some have determined the activity in whole brain while others have opted for discrete brain areas and nuclei. Brown, Nicholass and Redfern (1982) did not find any rhythm in synaptosomes prepared from whole rat brain. Similarly Deguchi (1977), and McLennan and Lees (1978) had earlier reported the absence of any rhythm in the rat pineal and midbrain and striatum respectively. The latter group did however

detect a significantly higher Km for BH_4 during the subjective daytime.

However, Kan et al., (1977) found a significant rhythm in the striatum and raphe nuclei of the rat. Similar results were reported in mice by Natali et al., (1980) and in the rat brainstem by Cahill and Ehret (1981). The rhythm has also been demonstrated in the pineal enzyme (Toru et al., 1979; Shibuya, Toru and Watanabe, 1978). In all cases where the rhythm was observed, the activity was found to be significantly higher in the dark than in the light period.

On balance then, it would seem that there is a good case for a circadian control in the activity of Try-OHase. The mechanism for generating this rhythm, however, remains to be elucidated; though there is evidence to suggest that in the pineal gland it is regulated by the noradrenergic system which induces increased synthesis of the enzyme in the dark period (Toru et al, 1979; Sitaram and Lees, 1978). This problem is addressed to further in Chapter 5.

2.3. ASSAY OF TRYPTOPHAN HYDROXYLASE

The method used for assay of Try-OHase was adapted from that of Ichiyama and his colleagues (Ichiyama et al, 1970). It involves the use of LTRY radio-labelled at the carboxyl side chain. The reaction is illustrated as follows, with the labelled ^{14}C atom on the carboxylic acid group (*).

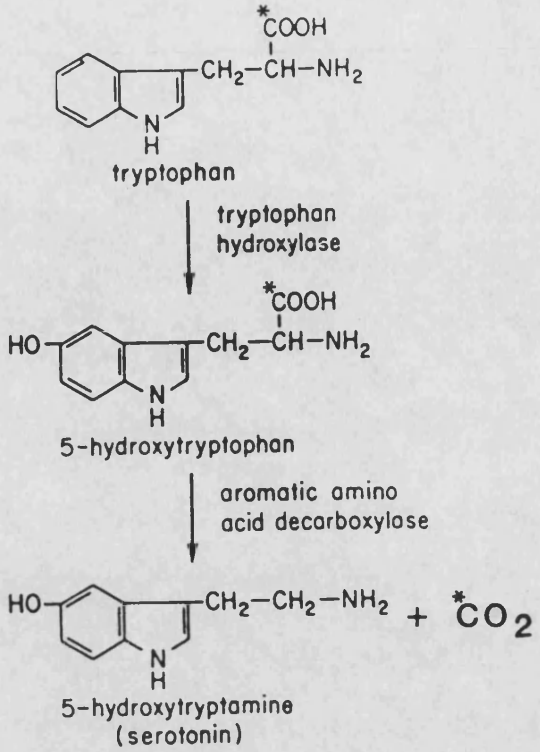


Fig. 2.3 The metabolism of [^{14}C] L-Tryptophan.

$^{14}CO_2$ released at the decarboxylation step is trapped and counted by a scintillation counter.

This method exploits the fact that 5HTP decarboxylase has a much higher affinity for 5HTP than L-TRY, the respective Km's being $2x10^{-6}$M and $3x10^{-3}$M (Lovenberg et al, 1962). Thus in a mixture of the two amino acids with low concentration of L-TRY, 5HTP is prefentially decarboxylated.

Since the specific activity of 5HTP decarboxylase is much

greater than that of Try-OHase (70-100 times) any hydroxylated L-TRY is immediately decarboxylated, so that in presence of an excess of 5HTP decarboxylase no 5HTP accumulates. The number of moles of $^{14}CO_2$ collected therefore corresponds to the number of moles of labelled L-try and in effect gives an index of the activity of Try-OHase. This assumption has been inferred from the stoichiometry of the L-TRY hydroxylation which has been determined (Friedman, Kappelman and Kaufman, 1972) as: L-TRY + BH_4 + O_2 ——> 5HTP + $QDHBH_2$ + H_2O.

The success of the assay depends on the concentration of L-TRY being kept very low (below 20 μM) otherwise at higher concentrations, significant decarboxylation of L-TRY to form tryptamine occurs (Ichiyama et al, 1970). It is therefore worth pointing out at the outset that this method is not suitable for studying the activity of Try-OHase at saturating concentrations of L-TRY. Another drawback with this technique is that it cannot be applied to crude enzymatic extracts from tissues such as the liver where other pathways of L-try metabolism exists.

Other techniques are available for assay of TRY-OHase. The most direct method involves the measurement of 5HTP which accumulates when a decarboxylase inhibitor is included in the reaction mixture. 5HTP can then be detected fluorometrically (Friedman, Kappelman and Kaufman, 1972) or by the new techniques of High Pressure Liquid Chromatography with electrochemical detection. Other investigators have used L-Try radiolabelled with ^{14}C at the side chain and then isolated radiolabelled 5HTP formed by thin layer chromatography (Kan et al, 1977). These and other techniques have been reviewed by Boadle-Biber (1982).

2.4. Properties of the synaptosomal tryptophan hydroxylase

When brain tissue is homogenized and centrifuged according to the method of Gray and Whittaker (1962, reviewed by De Roberties, 1967) the sheared-off membranes at the nerve endings reseal entrapping part of the cytosol to form synaptosomes. It contains part of the soluble cytoplasmic components such as lactate dehydrogenase, pyridine nucleotides, small molecular weight ions such as K^+, amino acids, neurotransmitters etc. The synaptosome is capable of high respiration and a wide range of biosynthetic reactions (reviewed by De Belleroche and Bradford, 1973).

It has been demonstrated by several investigators that synaptosomes contain tryptophan hydroxylase. About 40-70% of the activity is associated with it while the rest, the so-called soluble enzyme, is retained in the supernatant fraction (Graham-Smith, 1967). The synaptosome is analogous to the in situ situation and thus the assay of Try-OHase does not require any addition of exogenous pteridine cofactor, $NADH_2$ or reducing agents. The enzyme in the whole homogenate behaves likewise. On the other hand the activity in the supernatant fraction or a partially purified enzyme shows an absolute dependence on exogenous cofactor, and $NADH_2$ and dihydropteridine reductase. The reducing system could be replaced by high concentration of sulfhydryl compounds e.g. mercaptoethanol or dithiothreitol. Catalase or Fe^{2+} is also included to reduce H_2O_2 formed from side reaction which would otherwise accumulate and inhibit the enzyme (Ichiyama et al, 1970; Friedman, Kappelman and Kaufman, 1972; Lovenberg, Jequier and Sjoerdsma, 1968; Graham-Smith, 1967).

Try-OHase in the synaptosome, alias the "particulate" enzyme,

can be released when the preparation is subjected to an osmotic shock, after which it behaves like the soluble enzyme, its activity reverting to complete dependence on the exogenous cofactor and the reducing system (Graham-Smith, 1967). This observation led Graham-Smith and his associates to doubt the notion of the "soluble" and "particulate" sub-types of the enzyme (Green and Graham-Smith, 1975). They hold the view that the only difference is due to the technique of preparation.

Throughout this work synaptosomes or whole homogenates were used as a source of the enzyme. As explained above, the endogenous pteridine cofactor (BH_4) and $NADH_2$ ($NADPH_2$) are retained intact in these preparations and hence the need to add exogenous forms is obviated.

2.5. CABINETS AND ANIMAL MAINTENANCE

Specially constructed cabinets were used to house the rats. Domestic extractor fans (Phillips, Type HR 3408) were fitted to draw a costant current of air through the cabinets. One fan served four cabinets. Lighting was supplied in each cabinet by a small white 8 watt fluorescence tube (Phillips TL 8W), with the choke removed and refitted outside the cabinet to prevent overheating. Each light was connected through a time switch (Sangamo Time Controls). Plastic foam strips (1.3 cm) were glued to the perimeter of the inner door to ensure light proofing. Sound insulation was improved by lining the inside of each cabinet by 1.3 cm polystyrene sheets. These cabinets had earlier been characterised and found to be adequate (Hillier, Davies and Redfern, 1973).

The animals were maintained on a 12 hr light: 12 hr dark

(LD12:12) cycle. Since the experiments were performed during the normal working hours in the laboratory, this necessitated some animals being phase-shifted. At least 14 days was allowed to entrain the animals to the new light-dark cycle before use in the experiments. When the animals had to be killed or injected in the dark phase, the laboratory was illuminated only by a photographic red lamp since it has been shown that red light does not have any significant effect on circadian rhythms (McGuire, Rand and Wurtman, 1973).

Food (CRM pellets, Labsure) was provided in each cabinet. An automatic watering system was supplied by tubes from a reservoir kept outside the cabinets. The animals had free access to both food and water.

The animals could be left undisturbed for several days, except when it was necessary to replenish the food and clean the cages. These conditions ensured that the animals were isolated from undue environmental interferences.

2.6. MATERIALS AND EXPERIMENTAL PROCEDURE

L-Tryptophan and methylbenzethonium hydroxide were purchased from Sigma Chem. Co. while L-[carboxyl-^{14}C]-tryptophan (specific activity 56 mCi/mmol) was purchased from Amersham International.

2.6.1. Dissection and Enzyme Preparation

Male Wistar rats (University of Bath strain) weighing between 80-120 g at the start of the experiment were housed in the cabinets as described above. After 14 days of entrainment they weighed between 200-300 g.

At different time points of the light:dark cycle, rats were decapitated, the brains removed and rinsed with ice-cold 0.32 M

sucrose solution. Dissection of the brain was carried out on a glass plate chilled on ice and lined by a filter paper moistened with the sucrose solution. The pineal gland, cerebellum and the olfactory lobes were discarded. The brainstem, striatum, hypothalamus and cerebral cortex were dissected out as described by Glowinski and Iversen (1966) and Miller et al., (1970). Briefly, this was done as follows (illustrated by the diagram below): a transverse section (1) was made separating the rhombencephalon from the rest of the brain. Another section was made at the limit of the anterior end of the hypothalamus through the optic chiasma (2). The hypothalamus was then removed as a rectangular block 2 mm deep. Section B of

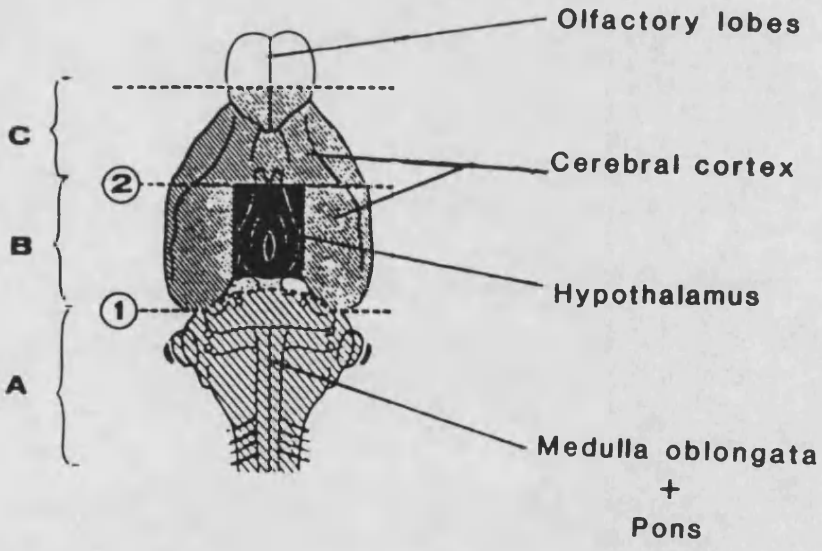


Fig. 2.6 The dissection of the rat brain

(Modified from Glowinski and Iversen, 1966).

the brain was turned on its ventral surface and the cerebral hemispheres pulled back and removed along with the corpus callosum and the striatum. Parts of the cerebral cortex and the striatum from section B were added to those from section C respectively. The brainstem therefore comprised of medulla oblongata, pons, thalamus and the midbrain. This dissection procedure was rapid and reproducible.

The dissected tissue was immediately blotted dry with soft tissue, weighed and homogenized in 0.32 M sucrose to form a 10% homogenate using a teflon pestle and glass tube homogenizer with 0.01" radial clearance. Usually about ten passes of the pestle were made. The glass tube was chilled in ice during homogenization. The homogenate was centrifuged at 1000 x g for 10 min at 4°C using MSE Chilspin (Fisons). The resultant supernatant was decanted and centrifuged further for 30 min at 12000 x g using MSE High Speed 18. The pellet so formed, the crude synaptosomal preparation (or P_2 fraction) was resuspended in a volume of 0.32 M sucrose solution to form a 10% suspension. The protein content of the preparation was determined by the method of Lowry et al., (1951).

2.6.2. The incubation mixture

The method of assay of Try-OHase used was adapted in this laboratory by Brown, Nicholass and Redfern (1982) from that of Ichiyama and his colleagues (as described above). Tris-acetate buffer pH 8.1 (50 μmol), 125 μmol sucrose and 0.1 ml of the enzyme preparation were pipetted into a narrow-necked bottle (Clinbritic, Mark II, 15 ml). The mixture was preincubated for 15 min at 37°C in a shaking water bath (Grant Instruments, model, SE15). 2.5 nmol L-Try

and 0.18 nmol (0.01 μCi) L-tryptophan carboxyl-^{14}C were then added. A micro-test tube was placed into the bottle and immediately clamp-sealed. The total concentration of L-TRY was thus 5.36×10^{-6}M in a final volume of 0.5 ml. After 30 min of incubation the reaction was terminated by an injection of 1.0 ml 4% perchloric acid into the bottle. The injection of the acid also served to release from solution the CO_2 formed. At the same time 0.5 ml 0.1M hyamine hydroxide (methylbenzethonium hydroxide) in butan-1-ol was injected into the micro-test tube. Hyamine is toxic to most biological systems and that is why it was added at the end of the reaction. The incubation was left in the shaking water bath at 37°C for a further 3 hr to allow for complete absorption of the released $^{14}CO_2$ by the hyamine. The blanks were treated likewise except that the enzyme was first pre-incubated in 4% perchloric acid for 20 min. Finally, the seal was removed from the bottle and hyamine transfered to a vial containing a scintillant (Aqua Luma, Lumac) in a final volume of 5.0 ml. The scintillation counter used was Rackbeta 1215 (LKB, WALLAC).

The incubation protocol described above was used to determine the circadian rhythm of the activity of Try-OHase in the brainstem, cortex and striatum of the rat brain. This was adopted as the most appropriate after a series of experiments had been done to determine how $^{14}CO_2$ evolved varied with the amount of enzyme, initial substrate concentration and time of incubation. The amount of each ingredient in the incubation mixture and the time of incubation are detailed in the results section. To prepare a 33% homogenate, brainstems were pooled from two rats so as to provide a volume enough for a series of incubations.

2.7. RESULTS

Figs 2(a)-2(e) show how the activity of the enzyme varied with time of incubation, initial concentration of L-try and the amount of the enzyme. Two sources of the enzyme were used in these preliminary assays: cortical synaptosomal preparation [figs 2(a) and 2(b)] and brainstem homogenates [figs 2(c)-2(e)]. The activity of the enzyme is expressed as the amount of total CO_2 formed, pmol/hr/mg protein of a 10% (or 33%) enzyme preparation. The vertical bars represent the standard errors of the mean (SEM). The n values are indicated in the legends.

The amount of CO_2 evolved measured against varying concentrations of L-Try was linear within the range of L-Try concentration used [fig 2(b) and 2(d)] and also with the amount of the enzyme [fig 2(e)]. Similarly it increased with time for the incubation period of 60 min [figs 2(a) and 2(c)].

The circadian variation of the enzymatic activity in the cortex, the striatum and the brainstem is represented graphically in fig. 2(f). The activity is expressed as total CO_2 evolved, pmol/mg protein of a 10% synaptosomal preparation/30 min. The vertical bars represent SEM; and n=3 or 4. The difference in activity between the dark and light periods was assessed statistically by analysis of variance and the following p values were obtained: corpus striatum, $p<0.001$; cortex, $p<0.01$ and the brainstem, $p<0.01$. These values therefore show that the activity of Try-OHase is higher in the dark phase than in the light phase of light-dark cycle and that the difference is statistically significant. These results therefore corroborate some of the literature reports cited in the introduction of this chapter. The significance of these findings are discussed in the discussion section, Chapter 5.

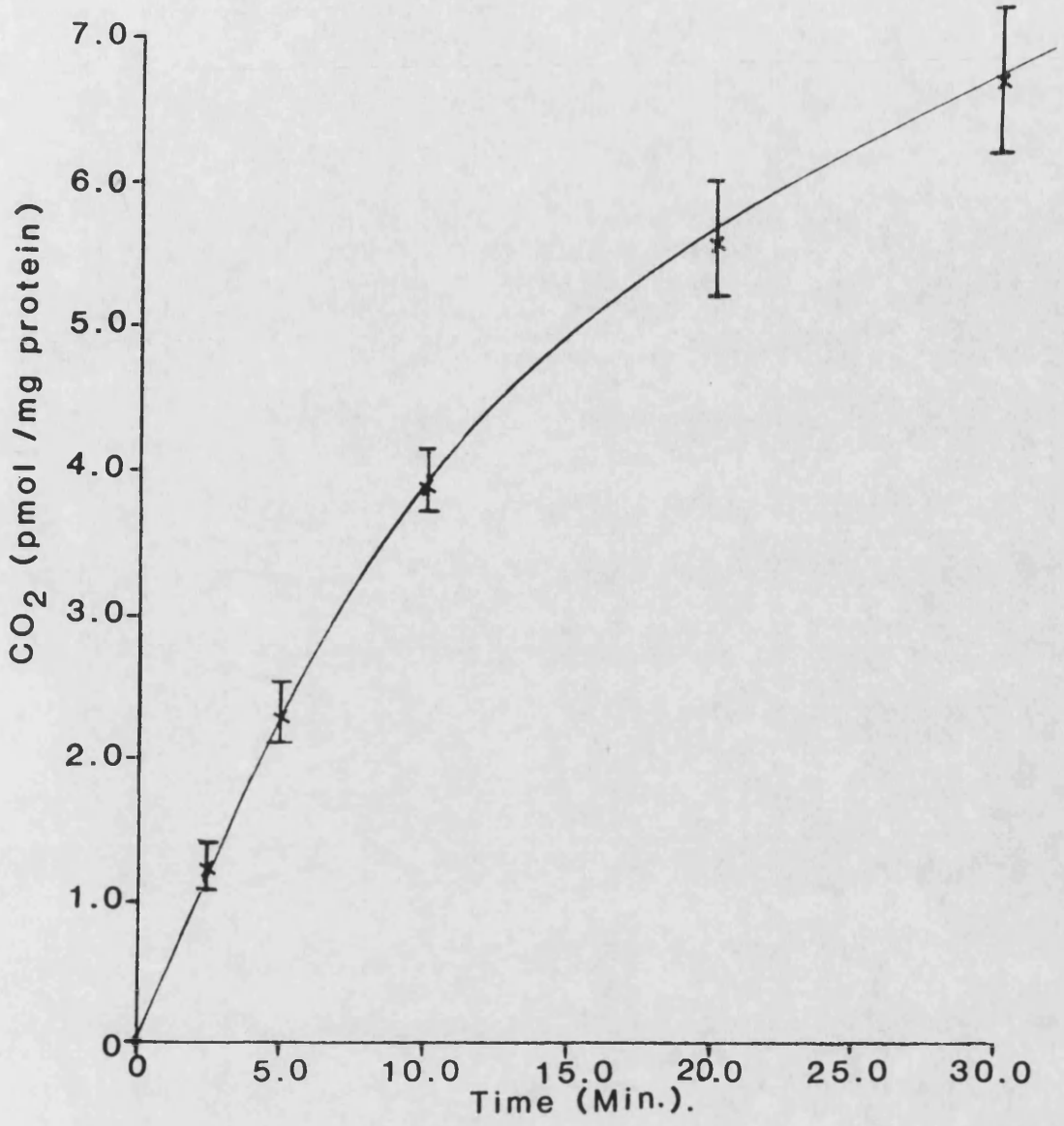


Fig. 2(a)

The activity of Try-OHase versus time in cortical synaptosomal preparation. The incubation mixture in each bottle contained: 0.1 ml enzyme preparation, 0.01 µCi (0.2 nmol) labelled L-try., 2.5 nmol unlabelled L-try., 125 µmol sucrose, 50 µmol tris-acetate (pH 8.1) in a total volume of 0.5 ml. Enzymatic activity is expressed as total CO_2 evolved, pmol/mg protein of 10% synaptosomal preparation $\pm$ SEM; n=3.

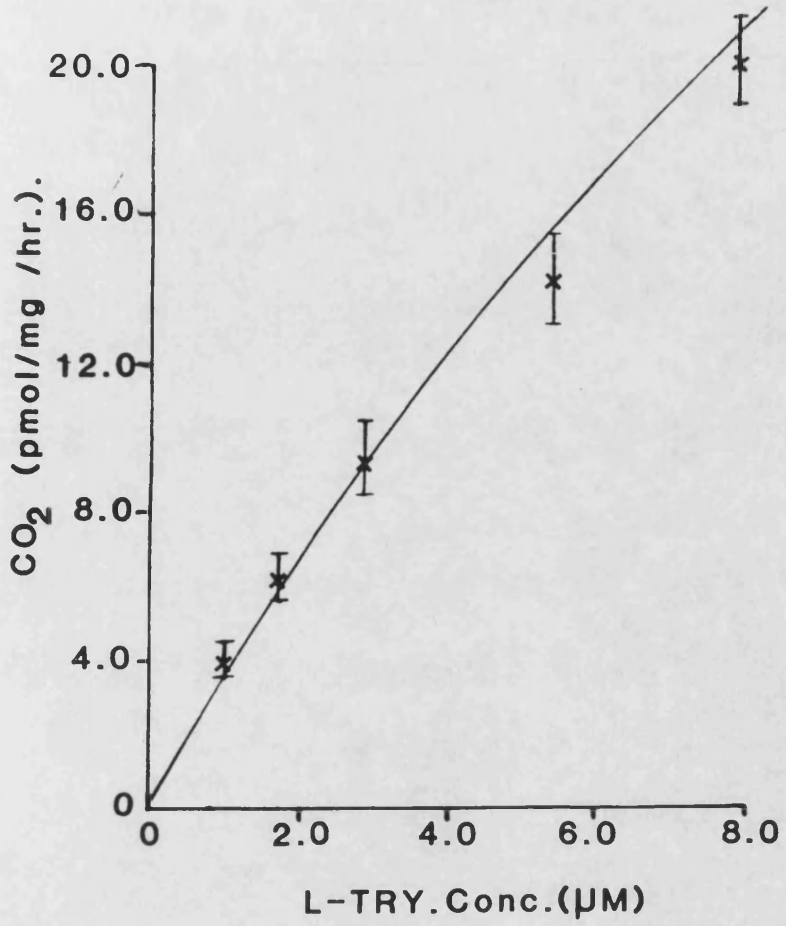


Fig. 2(b).

The activity of Try-OHase versus substrate concentration in cortical synaptosomal preparation. Each incubation mixture contained the same ingredients as shown under fig. 2(a) except: unlabelled L-try, 0.3-3.75 nmol and incubation time of 60 min. The enzymatic activity is expressed as CO_2, pmol/mg protein of 10% synaptosomal preparation/hr, $\pm$ SEM; n=4.

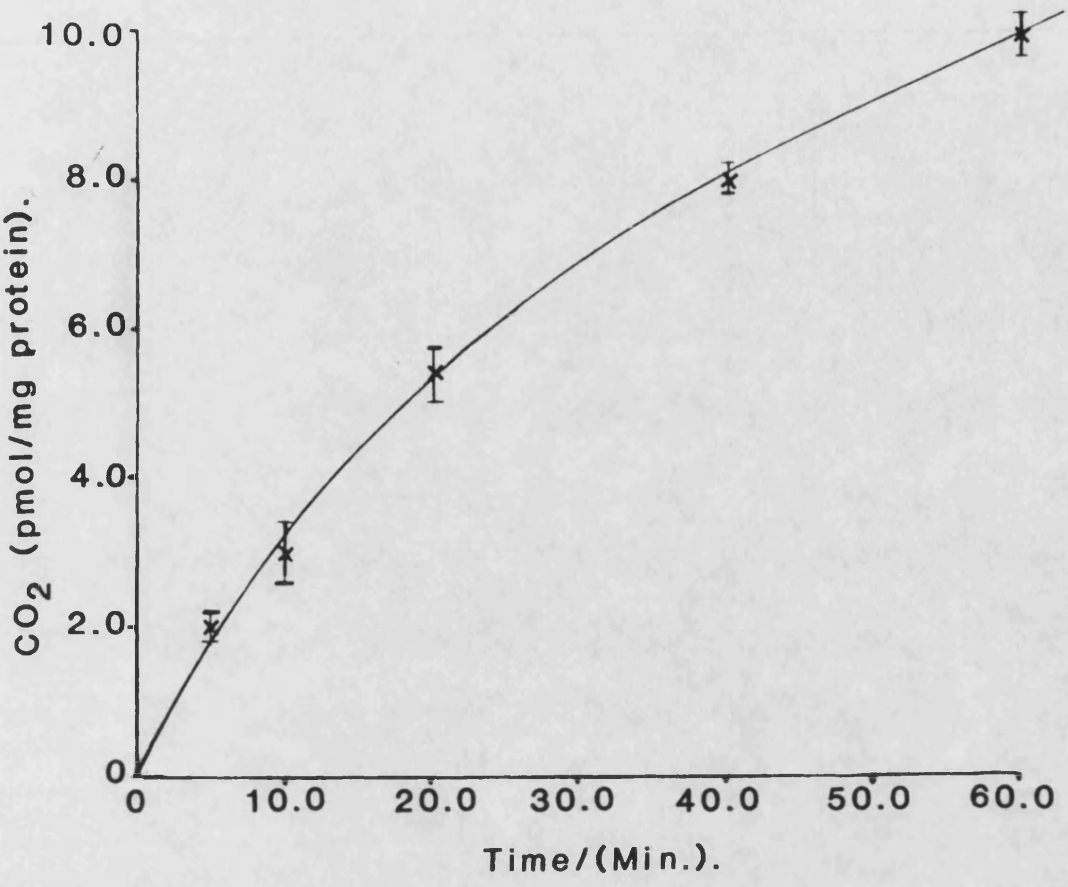


Fig. 2(c).

The activity of Try-OHase versus time in 33% brainstem homogenate. For conditions of incubation, see fig. 2(a). The activity of the enzyme is expressed as CO_2, pmol/mg protein of 33% homogenate/hr, $\pm$ SEM; n=4.

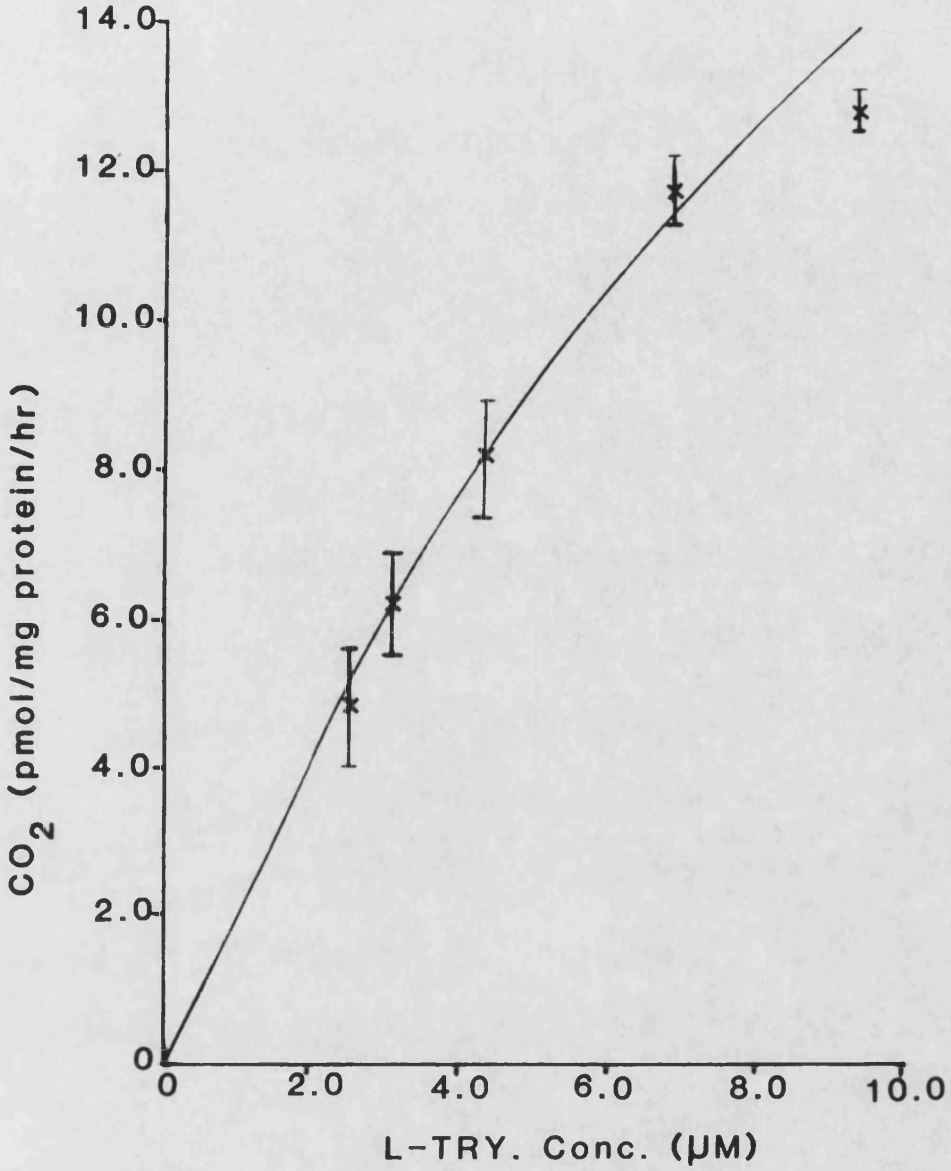


Fig. 2(d).

The activity of Try-OHase versus L-try concentration in brain-stem homogenate (33%). For conditions of incubation see fig. 2(a). L-Try concentration varied from 1.0-3.75 nmol. The results are expressed as pmol/mg protein of 33% homogenate/ hr $\pm$ SEM; n=4.

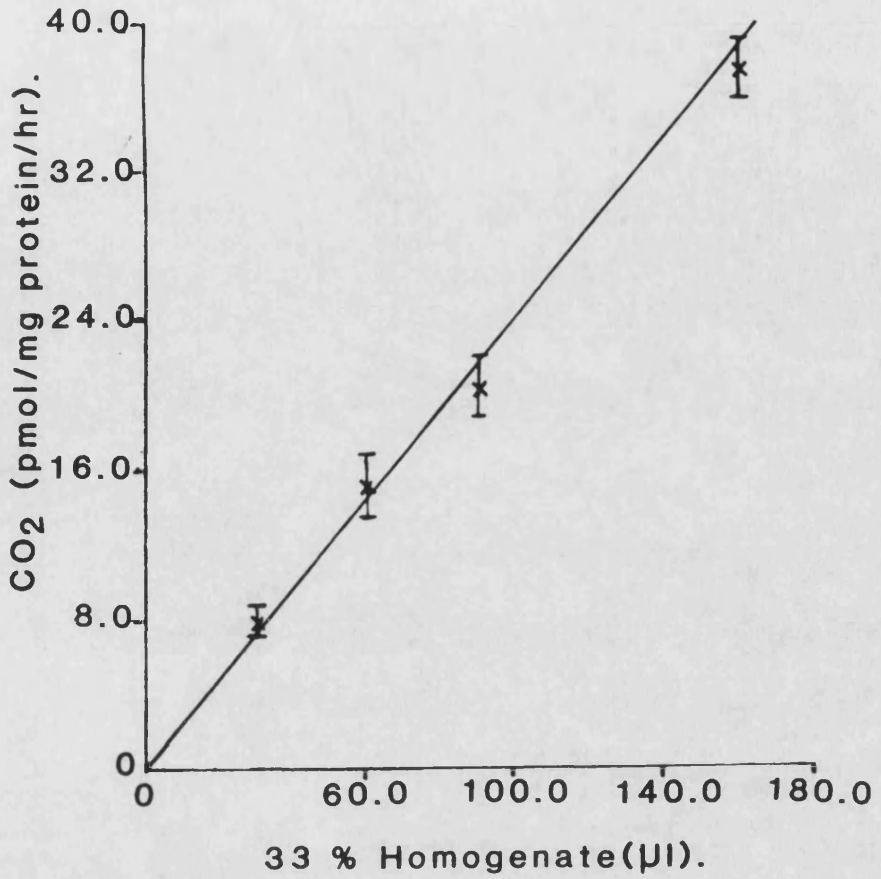


Fig. 2(e).

The activity of Try-OHase measured against varying amounts of brainstem whole homogenate. Each incubation mixture contained varying volumes of 33% homogenate, labelled L-try, 0.05 µCi (0.91 nmol) and other ingredients as in fig. 2(a). The incubation time was one hour. Results are expressed as CO_2, pmol/mg protein of 33% homogenate/hr, $\pm$ SEM; n=3.

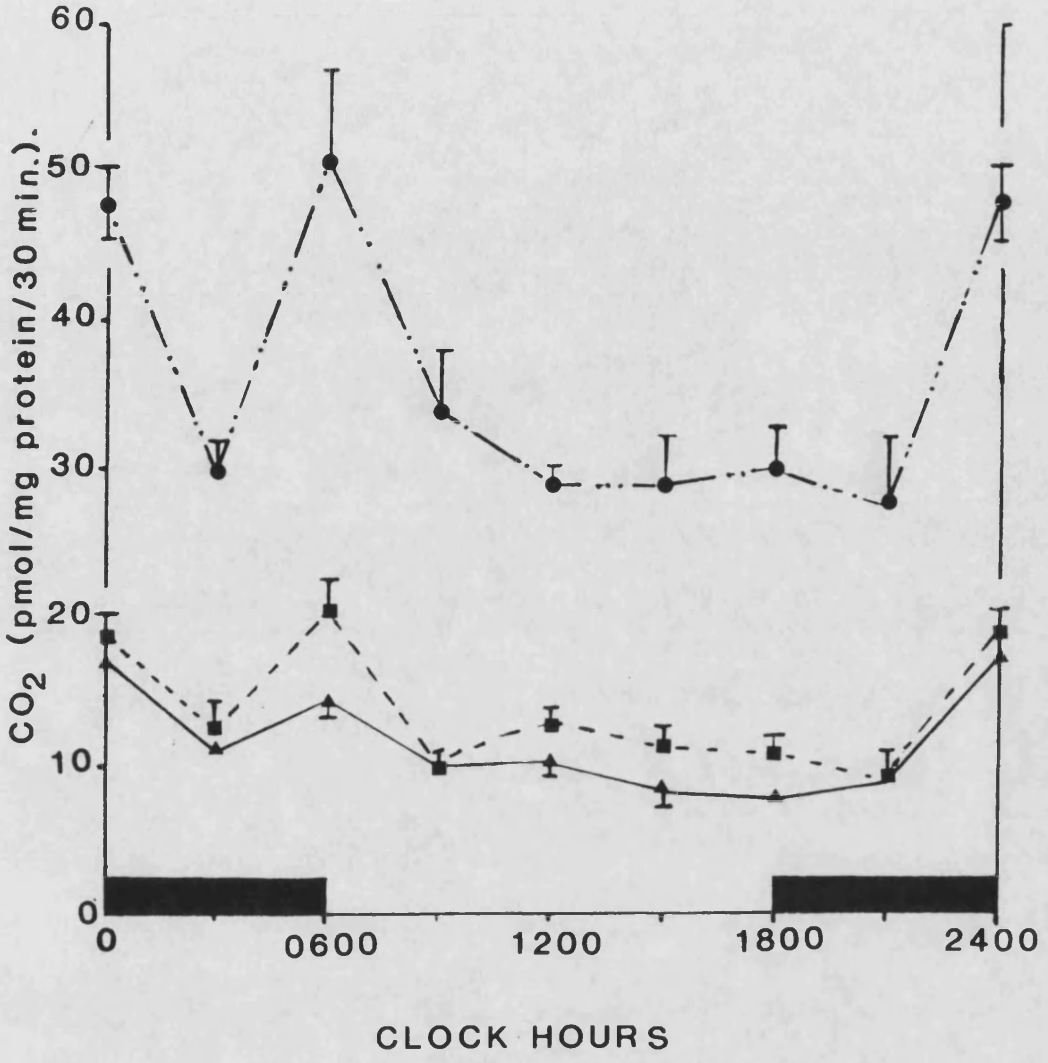


Fig. 2(f).

The circadian rhythm of Try-OHase activity in the rat striatum (●-··-●), the brainstem (■- - -■) and the cortex (▲——▲). The activity is expressed as CO_2, pmol/mg protein of 10% synaptosomal preparation/30 min ± SEM. The solid black bars represent the hours of darkness.

Chapter 3

3. IN VIVO ASSAY OF CIRCADIAN RHYTHM OF 5HT SYNTHESIS

3.1. INTRODUCTION

In vitro techniques have been used extensively to study the synthesis of 5HT. Although they have yielded invaluable information about the properties and kinetics of TRY-OHase, they fail to take into account physiological regulation which may come into play in a whole organism. The activity of the enzyme obtained in vitro is thus not necessarily equal to in vivo activity. The need to develop methods for in vivo assays was therefore necessary in order to understand the regulatory processes involved in the synthesis of 5HT (and other monoamines) and how this relates to functional state of the neurons.

Turnover rates of monoamines have been widely used as a measure of their rates of synthesis. Turnover is a dynamic process and refers to renewal of a substance in a tissue in a steady-state condition. This is accomplished in two different ways: synthesis of new substance within the tissue or synthesis elsewhere but transported to the tissue by circulation. Several assumptions have to be made in kinetic studies of turnover. One open compartmental model of the neurons is assumed whereby the newly synthesised molecules rapidly mix with the stored ones. It is also implied that a steady-state in the system exists. Thus synthesis and transport of monoamines into the system equals breakdown and exit from the system so that under normal circumstances the level of monoamines are kept relatively constant.

Since very little 5-HT enters the brain from peripheral circulation because of poor penetration of the blood-brain barrier, its turnover rate in the brain more or less reflects its rate of

synthesis there. Moreover, since it has been demonstrated that stimulation of serotonergic neurons increases turnover of 5HT, it appears to measure the functional state of the neurons (see section 3.3).

3.2. METHODS OF MEASUREMENT OF TURNOVER OF 5HT

The methods of measurement of turnover of 5HT fall broadly into two categories: steady sate and non-steady state methods. In the steady state techniques tracer amount of isotopes, too small to disturb the equilibrium are administered. Measurement of 5HIAA in urine and CSF also fall into this category. Non-steady state methods on the other hand involve administration of drugs to interrupt one point or the other in the synthetic or the metabolic pathway of 5HT i.e. at the hydroxylation, decarboxylation or deamination steps. The resultant accumulation or decline of one of the metabolites is then monitored, the assumption being that the rate of 5HT synthesis is not affected by the treatment, at least not initially. The steady state techniques offer the theoretical advantage that the system is not perturbed. A few examples of the two techniques are described below.

3.2.1. The steady state methods

(a) Labelling with [H^3]5HT

In this method, trace amounts of [H^3]-5HT are introduced intracerebroventricularly (Schildkraut et al, 1969). It is assumed that it rapidly and uniformly equilibrates with the endogenous pool of 5HT. The rate of decline of [H^3]5HT therefore gives a measure of

5HT turnover. There is evidence however, that the assumptions made are not always true. For example, labelled 5HT tends to concentrate more around the ventricles than the rest of the brain and it probably enters non-serotonergic neurons or glial cells; secondly, the rate of equilibration with endogenous 5HT is not always a rapid process (Weiner, 1974, quoted by Neckers, 1982). This technique is thus likely to give an inaccurate measure of whole brain turnover of 5HT.

(b) Labelling with [H^3]L-Try

This technique involves the introduction of labelled L-Try either as a bolus injection or intravenous infusion (Lin et al, 1969). The rate of formation of 5HT is monitored by measuring changes in specific activity of labelled 5HT. At the same time, changes in specific activity of [H^3]L-Try in brain tissue or plasma is measured. The rate of turnover of 5HT is then determined from these data. This method assumes that the specific activity of L-Try at the hydroxylation site is equal to that in brain tissue or in plasma. There is evidence that there are regional differences in rates of uptake of L-Try (Neckers et al, 1977). In addition, all cells in the brain (both neurons and glial cells alike) utilize L-Try is protein synthesis and therefore total tissue specific activity of L-Try does not necessarily reflect that in serotonergic neurons. Another assumption is that newly synthesised radioactively-labelled 5HT is not metabolized or lost from the issue. This assumption is most probably untrue.

(c) Labelling with $^{18}O_2$

When $^{18}O_2$ is substituted for $^{16}O_2$ in the inspired air, it

eventually gets incorporated into 5HT at the hydroxylation step of L-Try. A gas chromatograph-mass spectrometer detector can then be used to separate and measure [^{18}O]5HT and [^{16}O]5HT in CSF, blood or urine. From the relative abundance of $^{18}O_2$ to $^{16}O_2$, rates of turnover of 5HT can be calculated. This technique, used by Galli et al, (1977, 1978) on animals is potentially applicable to humans because $^{18}O_2$ is non-radioactive, easy to administer and is believed to be non-toxic.

(d) Measurement of 5HIAA

5-HIAA is a major metabolite of 5HT. It is secreted into cerebrospinal fluid (CSF) and finally cleared from the brain by an acid transport system (Neff, Tozer and Brodie, 1967). Measurements of steady-state concentrations of 5HIAA in CSF and urine have been used as an index of central 5HT turnover. It is below normal in CSF of subgroups of depressed patients.

Accuracy of turnover estimates using this method is limited by the assumptions which must be made. For instance, the 5HT/5HIAA ratio cannot be assumed constant in all brain areas because it is known that 5HT turnover rate in serotonergic cell bodies is 10-15 times higher than in the terminals (Neckers, 1982). Thus measurement of steady-state concentration of 5HIAA in CSF gives an underestimate of whole brain turnover, probably reflecting lower turnover in the terminals (Neckers, 1982). Secondly, one must also assume that the acid transport system is independent of the concentration of 5HIAA; that is, it is not rate-limiting. Nevertheless, this technique has been used successfully in many laboratories.

3.2.2. Non-steady state techniques

(a) Accumulation of 5HIAA following probenecid administration

Measurement of 5HIAA is employed differently in one form of non-steady state technique. This arose from the knowledge that probenecid blocks the acid transport system mentioned above, leading to an accumulation of 5HIAA in brain and CSF of many animal species including humans. Following peripheral administration of probenecid there is a dose-dependent rise in 5HIAA which is linear for about 90 min in rat brains. The initial increase in 5HIAA gives an index of 5HT synthesis. This technique is applicable to humans by measuring 5HIAA in CSF.

Among the problems with this technique is that probenecid tends to increase the concentration of L-Try in the brain (Van Wijk et al, 1979, quoted by Neckers, 1982). This would lead to increased 5HT synthesis (since Try-OHase is not normally saturated with L-try) which is converted to more 5HIAA. Since this process disturbs the equilibrium at another point other than the one intended, it would render the results invalid.

(b) Inhibition of monoamine oxidase by pargyline

Pargyline is an irreversible inhibitor of monoamine oxidase (MAO) and following its peripheral administration, there is a linear increase in brain 5HT concentration for about 90 min after which it then plateaus. 5HT turnover is estimated from the linear part of the curve. Among the assumptions made are: that for every mole of 5HT synthesised one is degraded by MAO, that MAO is inhibited almost immediately and completely; that none of the 5HT so synthesised is lost from the brain by diffusion and that metabolism of 5HT by other

pathways is negligible.

When MAO has been inhibited in this way, 5HIAA is no longer formed and its level in the brain declines exponentially. This provides yet another method of estimating 5HT turnover, assuming that the rate of efflux of 5HIAA is equal to its rate of formation.

(c) Inhibition of Aromatic amino acid decarboxylase (AAD)

5-Hydroxytryptophan decarboxylase (5HTP-DC) is also termed l-aromatic amino acid decarboxylase (EC4.1.1.28) because it shows activity towards dihydroxyphenylalanine (DOPA) and other aromatic amino acids as well. It has been purified from the kidney, adrenal gland and the liver (Lovenberg, Weissbach and Undefriend, 1962; Awapara, Sand and Hanly 1962 and Lancaster and Sourkes, 1972) and from the earlier studies by these investigators and others it was concluded that 5HTP-DC and DOPA decarboxylase (DOPA-DC) were one enzyme. However this view was later revised when Sims and colleagues (reviewed by Sims, 1974) published evidence showing that the two enzymes differed substantially with respect to optimum pH, temperature, substrate and cofactor (pyridoxal 5-phosphate) concentration, subcellular distribution and effect of intracisternal administration of 6-hydroxydopamine. One drawback to this argument soon emerged when the two enzymes were shown to cross-react immunologically, suggesting that they share many antigenic determinants (Christenson, Dairman and Udenfriend, 1972). Melamed, Hefti and Wurtman (1980), studying the effects of selective destruction of dopaminergic and serotonergic inputs to the striatum also concluded that AAD was a single protein. It therefore seems not

to have been clarified whether one is dealing with one or two enzymes.

Under normal circumstances, for reasons explained earlier, the concentration of 5HTP is practically undetectable in the brains of laboratory animals. However when 5HTP-DC is inhibited its concentration rises rapidly (Carlsson et al, 1972). Among the commonly used inhibitors is 3-hydroxybenzylhydrazine (NSD1015) which shares with other hydroxyphenylalkylhydrazines the ability to inhibit AAD both *in vivo* and *in vitro*. For NSD1015 the onset is rapid and a virtually total inhibition can be achieved at doses that are non-toxic to the animal. The initial rise in 5HTP is linear for about 30 min or so and this has been used widely to study 5HT turnover, subject to similar assumptions as discussed earlier. This technique was used here to study the turnover of 5HT and its practical details, are described later.

3.3. UTILITY OF TURNOVER STUDIES

It is difficult to judge which of the methods reviewed above provide the most reliable estimate of 5HT turnover. Obviously each will be limited by the assumptions stated to a greater or lesser extent. A survey of the literature shows that values obtained using different methods are broadly similar (e.g. Neckers and Meek, 1976). This not only strengthens their validity but suggests that the major assumptions made are probably true. Some of these methods, especially the measurement of 5HTP (following AAD inhibition) using high pressure liquid chromatography with fluorescence or electrochemical detection have been applied widely to study 5HT turnover in discrete brain nuclei after various types of pharmacological manipulation

(Johnson and Moore, 1983; Duda and Moore, 1985; Lookingland et al, 1986 and Boadle-Biber et al, 1983). The high sensitivity of this method makes it suitable for studying the effects at the neuronal level which could easily be missed if one examined gross brain areas. From such studies, some investigators have suggested that 5HTP accumulation can be used as an index of neuronal activity (Duda and Moore, 1985; Bourgoin et al, 1980, quoted by Lookingland et al, 1986).

3.4. HIGH PRESSURE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

The indole ring of L-Try and its metabolic derivatives oxidize readily in the presence of an oxidizing agent. The electron released can be measured as an electric current, which is proportional to the amount of the substance oxidized. This forms the basis of electrochemical detection, with positive graphite or carbon electrode acting as the oxidant. The potential applied can be varied to obtain optimum oxidation for each compound, thus enabling them to be identified. Due to an electron rich centre contributed by the hydroxyl group, the hydroxylated metabolites of L-TRY oxidize at lower potentials (about + 0.5 V) while a higher potential (about + 0.8 V) is necessary to release an electron from the nitrogen in the indole ring of L-Try.

Liquid chromatography often used in conjunction with electrochemical detection usually consists of column based ion exchange or reverse phase systems. Reverse phase systems consist of a nonpolar stationary phase and a polar mobile phase. The stationary phase is usually a hydrocarbon-bonded surface, commonly octadecyl

silyl bonded to silica (C-18 columns) while buffer/methanol mixture is a commonly used mobile phase. Under such a system, the more polar compounds with the least affinity for the hydrophobic stationary phase elute first. An ion pairing agent, commonly sodium octyl sulfonate, is sometimes added to the mobile phase. It retards the passage of the hydrophilic compounds down the column to differing degrees and thus aids separation. Reverse phase chromatography represents a superior system to ion exchange in terms of efficiency and versatility, and when used with electrochemical detection with appropriate pH, buffer and flow rates provides one of the most convenient, relatively cheap, rapid yet highly sensitive techniques for biochemical assays. Its technological development continues to be improved and it is likely to represent a powerful tool for the future of neurochemical studies.

The reverse phase, ion-pair chromatographic system used in this work is described in detail in the next section.

3.5. EXPERIMENTAL SECTION

3-Hydroxybenzylhydrazine dihydrochloride (NSD1015) and L-Try were purchased from Sigma Chem. Co.

3.5.1. The HPLC system

Column tubes (length 25 cm and diameter 4.6 mm) and column ends were obtained from HETP. Hypersil ODS 5 μm particle size (C-18) from Shandon, was made into a slurry with isopropanol and packed into the column using a Haskell pneumatic pump at 7000 psi. The packing solvent was hexane. The efficiency of the column was assessed using the equation:

$$N = 5.54\left(\frac{ta}{W_{\frac{1}{2}}}\right)^2$$

where, ta = retention time of peak,

$W_{\frac{1}{2}}$ = width of peak at ½ peak height,

N = no. of theoretical plates.

Only the columns with $N \geq 5,000$ were used.

The column was then connected to the rest of the chromatographic system which consisted of the following: LDC pump, Constametric Model III; electrochemical detector (amperometric) with working glassy carbon and reference Ag/AgCl electrodes (Bioanalytical Systems Inc., model LC-4A); LDC/Milton Roy Integrator, model CI-10; LDC/Milton Roy Printer, model SEK; Autosampler, Magnus Scientific, model M7110 and sample injection valve, Rheodyne Inc., model 7010 fitted with 100 µl or 50 µl injection loop.

Doubly polished and deionized water was prepared using Milli Q Water Systems (Millipore Corporation). This was used to prepare the mobile phase, which was made up of: 0.07 M sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), 0.25 mM octane sulphonic acid sodium salt, 0.1mM EDTA and Methanol, 6-8%. The pH was adjusted to 4.0 with 1M perchloric acid. It was vacuum-filtered through 0.45 µm membrane filters (Whatman) and degassed by bubbling helium gas through for 20 min. For the assay of L-Try, 2.9 mM Sodium acetate (trihydrate) was included in the mobile phase.

The mobile phase was pumped through the detector for at least two hours to allow the detector current to reach normal baseline before the samples were injected. Usually the system was left pumping overnight at a reduced flow rate. Fresh buffer was prepared each day.

The detector was set at potential +0.65 V and range 5nA or 10nA and the pump flow rate was 1.7ml/min at 2000 psi. For L-Try assay, the detector was set at +0.75V and range 10 or 50nA. 5HTP and L-Try were identified from the retention times of authentic samples.

5HTP and dihydroxybenzylamine (DHBA) standards were prepared in deionized (Milli Q) water to an accurate concentration of about 200 µg/ml and stored frozen in sealed ampoules at -20°C and used within one month. Each time an assay was done, an ampoule of each standard was thawed and diluted 1:2500 with Milli Q water and used to calibrate the integrator. The diluted DHBA solution also served as internal standard in tissue extracts.

3.5.2. Routine Maintenance of the HPLC system

At the end of each day the column was cleaned by pumping a mixture of filtered Milli Q water and Methanol, HPLC grade (10:90) for 15 min, followed by only methanol. After about one week the top end of the column was detached and the top of the column inspected. Usually if there was a dark deposit it was scraped off and refilled with a freshly prepared slurry of hypersil ODS in methanol. The pre-column wire mesh was also cleaned in a sonicleaner (Dawe, Ultrasonics Ltd). The pump was cleaned about once a month by pumping warm 30% decomatic detergent (Decon Laboratories) at 8 ml/min for 4 min followed by water/methanol mixture and finally, methanol. When the sensitivity of the column had deteriorated considerably, it was replaced by a newly prepared one. Occasionally, the detector working and reference electrodes were wiped clean with a moist soft tissue.

When routinely maintained in this way, the HPLC system was

relatively trouble-free.

3.5.3. Preparation of the samples

Male Wistar rats were used throughout unless otherwise indicated. They were killed by decapitation and the brainstem and cortex dissected out as described in sec. 2.6.1. The brain regions were immediately placed in small sealable plastic sachets and kept frozen in liquid nitrogen till assayed. This was usually done the same day or if not then not later than one week.

The brain areas were weighed frozen and homogenized in 0.1 M perchloric acid in Milli Q water containing a known concentration of the internal standard, DHBA. The homogenate (10% w/v) was transferred to centrifuge tubes, shaken for one min in a whirl mixer (Fisons) and centrifuged at $0^{o}C$ and 15,000 x g for 15 min using MSE High Speed 18 ultracentrifuge. The clear supernatant was filtered by injecting through 0.2 or 0.45 μm pore size filters (Acro LC 13, Gelman Sciences). It was stored in ice and immediately injected into the HPLC system for assay. Protein content of the homogenate was assayed by Coomasie Blue method (Read and Northcote, 1981).

3.5.4. Time-dependent accumulation of 5HTP following 5HTP-DC inhibition

A dose of 100 mg/kg of NSD1015 in normal saline was injected i.p. to groups of rats weighing 150-220g which were then sacrificed 10,20,40,60 and 90 min later. This was done at the same time each day. The brainstem was isolated and 5HTP assayed as described above.

3.5.5. Effect of different doses of NSD1015 on 5HTP formation

Male Wistar rats weighing 175-225 g were used. 25,50,75 and 100

mg/kg doses of NSD1015 in normal saline were injected to different rats. They were sacrificed after 60 min and 5HTP assayed in the brainstem.

3.5.6. Differences between mid-dark and mid-light turnover of 5HT

From the preliminary assays above, a dose of 75 mg/kg and a period of 60 min between dose administration and sacrifice were selected as the most appropriate conditions and were used for the remaining part of the experiments unless otherwise indicated.

At the end of the entrainment period of at least two weeks to the light-dark cycle, the rats weighed 250-400 g. NSD1015 was administered (75 mg/kg, i.p.) at midnight or mid-day of the light-dark cycle and killed 60 min later. 5HTP was assayed in the brainstem and the cortex as before . This was also done in whole brains of another group of rats.

3.5.7. Estimation of <u>in vivo</u> Km of Tryptophan hydroxylase

(a) Variation of brain L-Try with time following a single i.p. dose of L-Try

This was done using female Wistar rats (University of Bath strain) weighing 190-250g. L-Try was dissolved in distilled water made to pH 8.0 with dilute NaOH. A dose of 100 mg/kg was injected i.p. to each rat. The rats were killed 20,40,80 and 120 min later and L-Try assayed in the brainstem and cortex. A group of control rats received only the vehicle and served as time 0 min.

(b) Variation of brain 5HTP and L-Try with doses of L-Try i.p.

Female Wistar rats weighing 180-250 g received 0,25,50,100 and 200 mg/kg doses of L-Try i.p. NSD1015 (75 mg/kg, i.p.) was administered 15 min later. The rats were killed 45 min later (i.e. 60 min after administration of L-Try) and 5HTP and L-Try assayed in the cortex and brainstem. Again the experiments were done the same time each day.

3.6. RESULTS

Fig 3(a) represents a typical chromatogram obtained with samples from rats treated with NSD1015. No 5-HTP was detectable in samples from rats that did not receive NSD1015 (results not shown).

Fig 3(b) shows the rate of accumulation of 5HTP in the brainstem following NSD1015 administration (100 mg/kg i.p.). The formation of 5HTP (and hence it is assumed the synthesis of 5HT) was linear with time for about 60 min (under the conditions of these experiments) after which it started to fall. This could be due to one or both of two factors: the action of NSD1015 on 5HTP-DC is wearing off and the enzyme is regaining its activity or 5HTP formed is leaking out of the brain into the periphery. In subsequent experiments, the rats were sacrificed 60 min after the dose of NSD1015.

The rate of 5HTP formation was also dependent on the dose of NSD1015, as depicted in fig 3(c). It was linear with doses below 50 mg/kg. As the dose rose to 75 mg/kg and above, no further increase was observed. Practically no 5HTP was detectable without the administration of NSD1015. A dose of 75 mg/kg was therefore used in

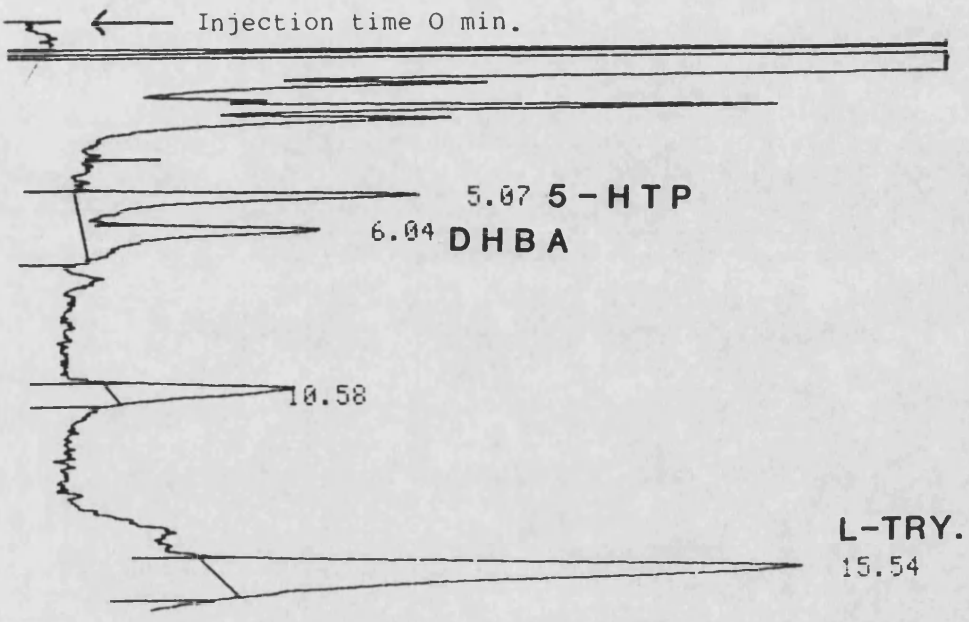


Fig. 3(a).

A typical chromatogram of the rat cortical extracts following a dose of NSD 1015 (75 mg/kg) 15 min after L-try (25 mg/kg). The detector potential was set at+O.75 V and range 10 nA. The pump flow rate was set at 1.7 ml/min. The mobile phase and other conditions of the experiment are described under "Experimental" section. 5-HTP: 5-Hydroxytryptophan; DHBA: Dihydroxy-benzylamine; L-Try: L-Tryptophan. The retention times are shown in mins.

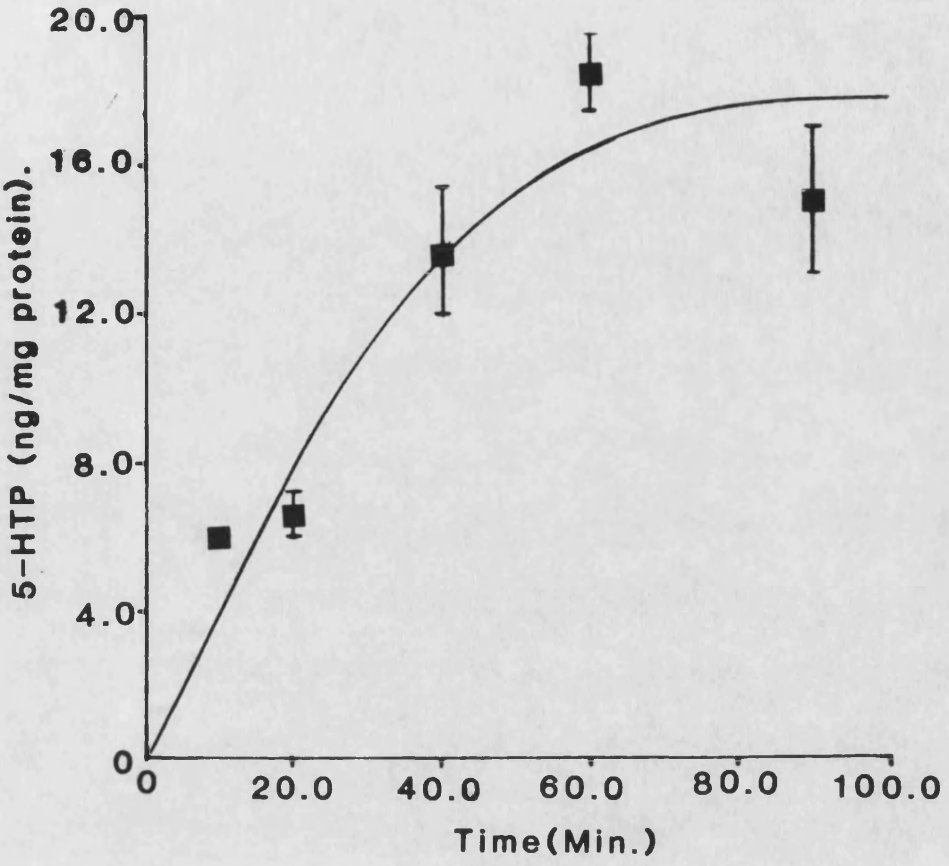


Fig.3(b).

5-HTP accumulation in the rat brainstem following a single peripheral dose of NSD 1015 (100 mg/kg i.p.). 5-HTP formed is expressed as ng/mg protein of a 10% homogenate of brainstem. The vertical bars represent SEM and n=5.

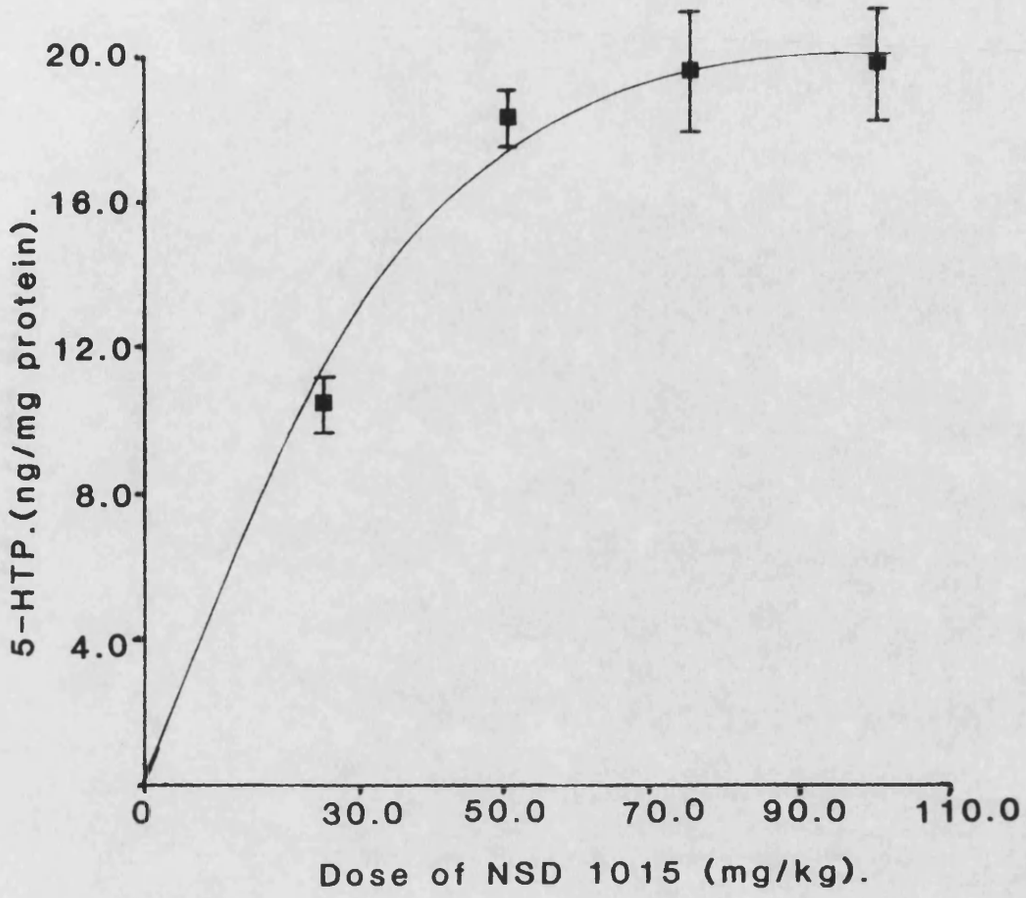


Fig. 3(c).

The effects of varying doses of NSD 1015 (i.p.) on 5-HTP accumulation in the rat brainstem. 5-HTP formed is expressed as ng/mg protein of a 10% brainstem homogenate/60 min ± SEM; n=3.

subsequent experiments.

Fig 3(d) compares the rates of 5HT synthesis between mid-dark and mid-light in the brainstem and cortex respectively. It is significantly higher in the dark period, with p values being <0.01 (brainstem) and < 0.001 (cortex). When the two brain areas are compared, the synthetic rate is similar in the dark period but significantly higher (p <0.001) in the brainstem than in the cortex in the light period.

When the synthesis of 5HT was compared between the dark and light period in the whole brain no statistically significant difference was detectable (Table 3). This was a surprising finding in view of the earlier observation that the cortex and the brainstem both had higher activities in the dark (above). The significance of this and the other results is discussed later.

Following a peripheral dose of L-Try (100 mg/kg i.p.) there was a rapid increase in L-Try concentration in both the brainstem and the cortex, reaching a maximum peak in about 60 min [fig 3(e)]. The decrease was equally rapid for it was approaching control values by 120 min. Of the two brain areas,the cortex had a higher rate of accumulation of L-Try.

Fig 3(f) shows the variation of brain L-Try and 5HTP following different doses of L-Try administered peripherally (0,25,50,100 and 200 mg/kg respectively). Again, the cortex had a higher rate of accumulation of L-Try than the brainstem at all the different peripheral doses of L-Try injected. However, as can be seen from the graphical representation, the ability of the brainstem to synthesize 5HT was more than double that of the cortex for the

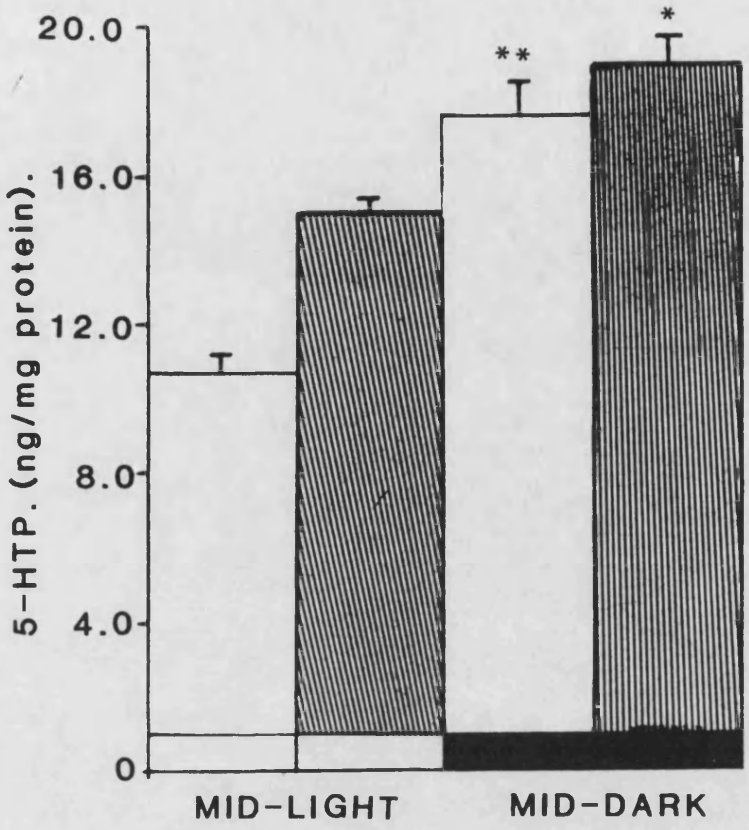


Fig. 3(d).

Differences between mid-dark and mid-light turnover of 5-HT in the rat brainstem (hatched columns) and cerebral cortex (open columns). The results are expressed as 5-HTP formed, ng/mg protein of a 10% homogenate/60 min. ± SEM and n=5 or 6. The solid black bar represents the mid-dark period of LD 12:12. * $p<0.01$, ** $p<0.001$ compared to mid-light values (Student's t-test).

MID-LIGHT	MID-DARK
5-HTP (ng/mg wet wt).	5-HTP (ng/mg wet wt).
0.277	0.305
0.340	0.347
0.300	0.301
0.325	0.381
0.296	0.320
	0.347
mean: 0.308	mean: 0.334
SEM: ±0.01	SEM: ±0.01

Table 3. Comparison of mid-light and mid-dark turnover of 5-HT in the rat whole brain.

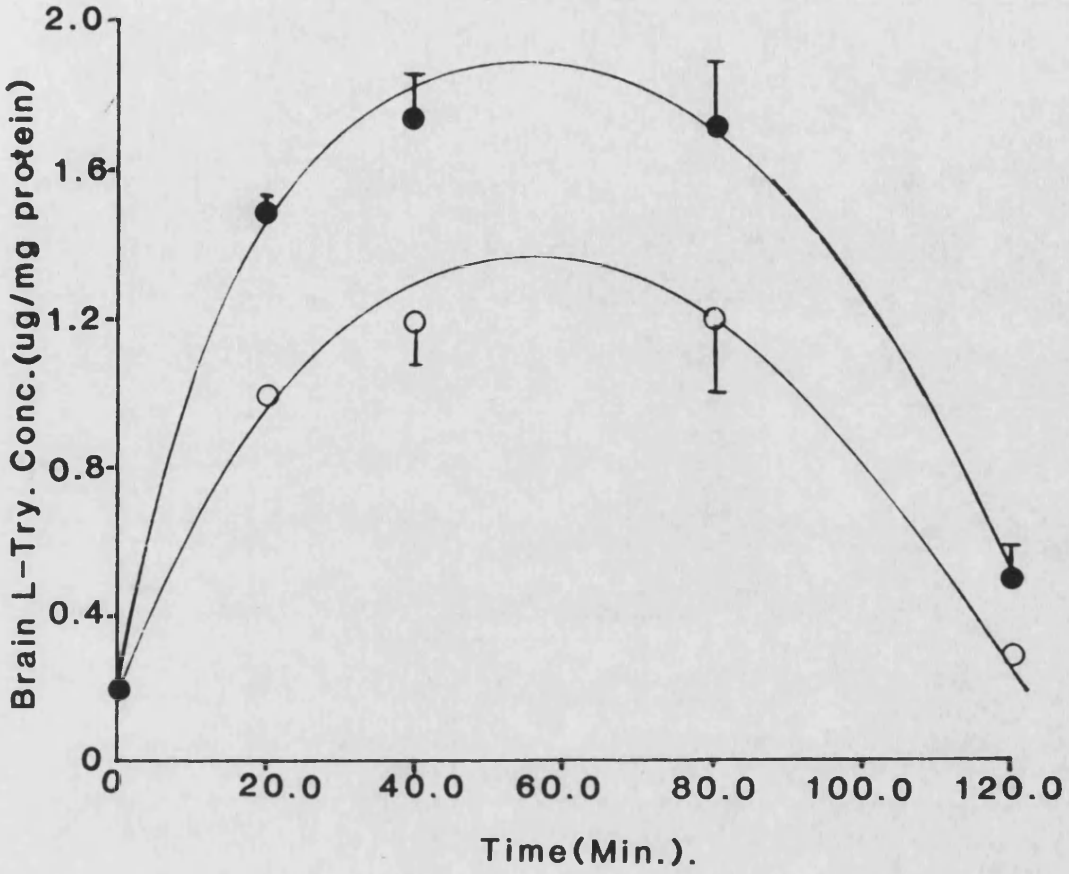


Fig. 3(e).

The variation of brain L-try with time following a single peripheral dose of L-try (100 mg/kg i.p.). L-try is expressed as µg/mg protein of a 10% homogenate of brainstem or cortex ± SEM and n=3. ●——● , cortex; ○——○ , brainstem.

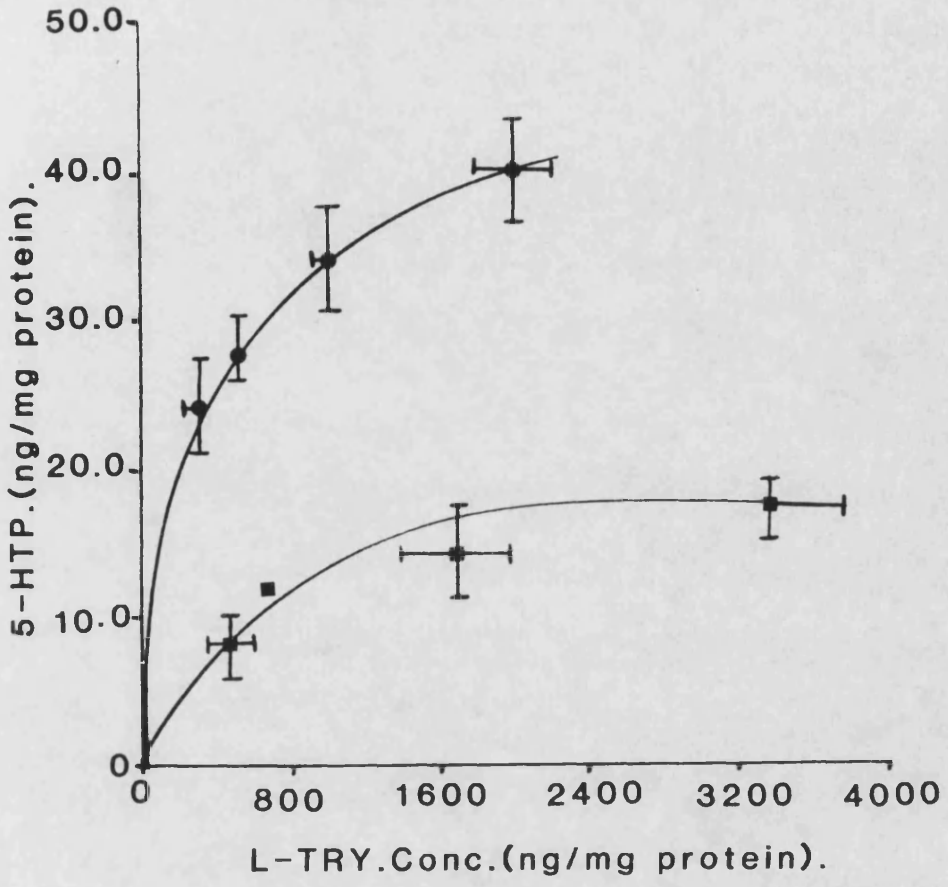


Fig.3(f).

The variation of 5-HTP and L-try in the rat cerebral cortex (■———■), and the brainstem (●———●) following different peripheral doses of L-try. Vertical and horizontal bars represent SEM of brain 5-HTP and L-try concentrations respectively, expressed as ng/mg protein of a 10% homogenate of the brainstem or the cortex.

corresponding doses of L-Try. The increase in 5HT synthesis by exogenous L-Try also confirms that L-Try-OHase is normally unsaturated *in vivo* with L-Try.

Double reciprocal plots of the effects of L-Try on the *in vivo* activity of Try-OHase are represented in figures 3(g) and 3(h). The following apparent kinetic parameters were calculated from Try-OHase in the brainstem: Km, 56 μM, V_{max}, 1.8 μg/g and the cerebral cortex: Km, 130 μM and V_{max} 0.8 μg/g. These results provide an evidence for regional differences in kinetics of Try-OHase. These values compare favourably with those in the literature. Carlsson et al., (1972) using a similar technique to the one used here to determine Km of mouse brain Try-OHase for L-Try reported a value of 50 μM. Values that have been obtained *in vitro* in the presence of BH_4 range from 14 μM (Kuhn et al., 1980b). 50 μM for a partially purified rabbit hindbrain enzyme (Friedman, Kappelman and Kaufman, 1972) to 120 μM for rat forebrain synaptosomal enzyme (Knowles and Pogson, 1984). Values as high as 300 μM have been reported in the presence of synthetic forms of pteridine cofactors (Lovenberg, Jequier and Sjoerdsma, 1968).

In vitro Km for L-Try was not determined here. Because of high risk of non-enzymatic decarboxylation the concentration of L-Try was kept below 25 μM (Ichiyama et al., 1970). This concentration is unlikely to saturate the enzyme.

The results from this chapter have shown that the *in vivo* rate of synthesis of 5HT is higher in the dark than light period, both in the brainstem and the cortex. These regions represent the mainly serotonergic cell-body rich areas and the nerve terminals respectively. These results corroborate those obtained in chapter two

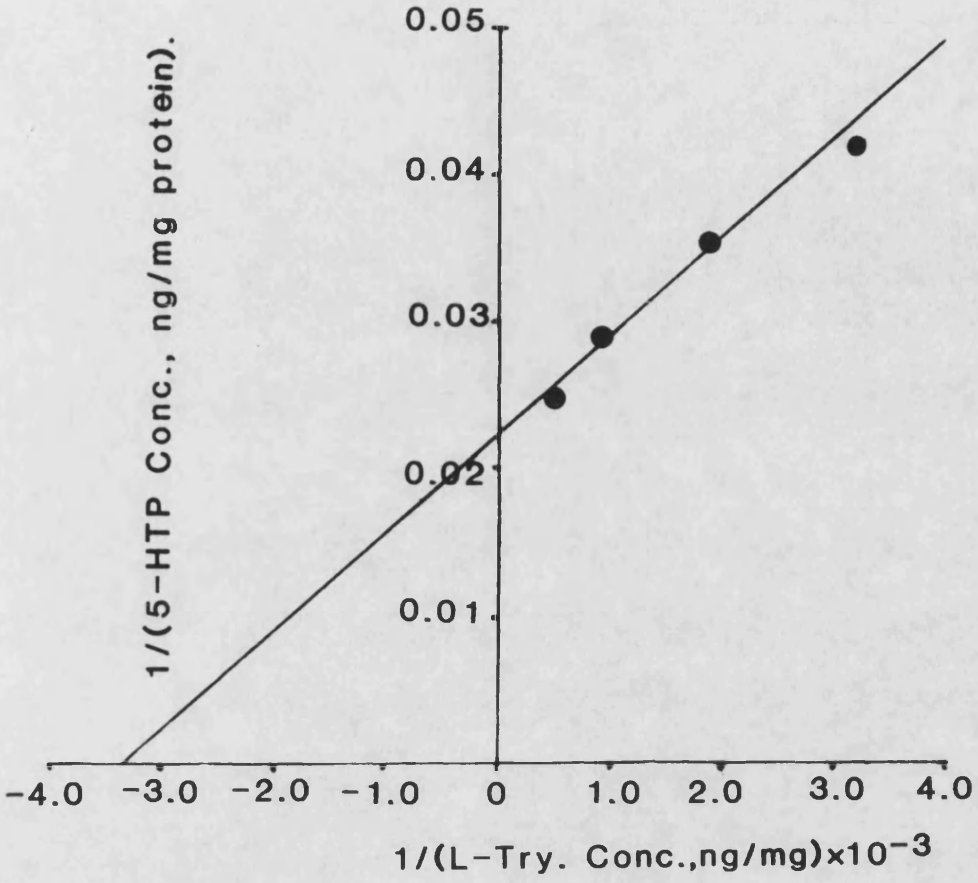


Fig. 3(g).

Lineweaver-Burk plot of the effect of L-try on the in vivo activity of Try-OHase in the rat brainstem. Km= 56 μM and Vmax =1.8μg/g of brainstem tissue.

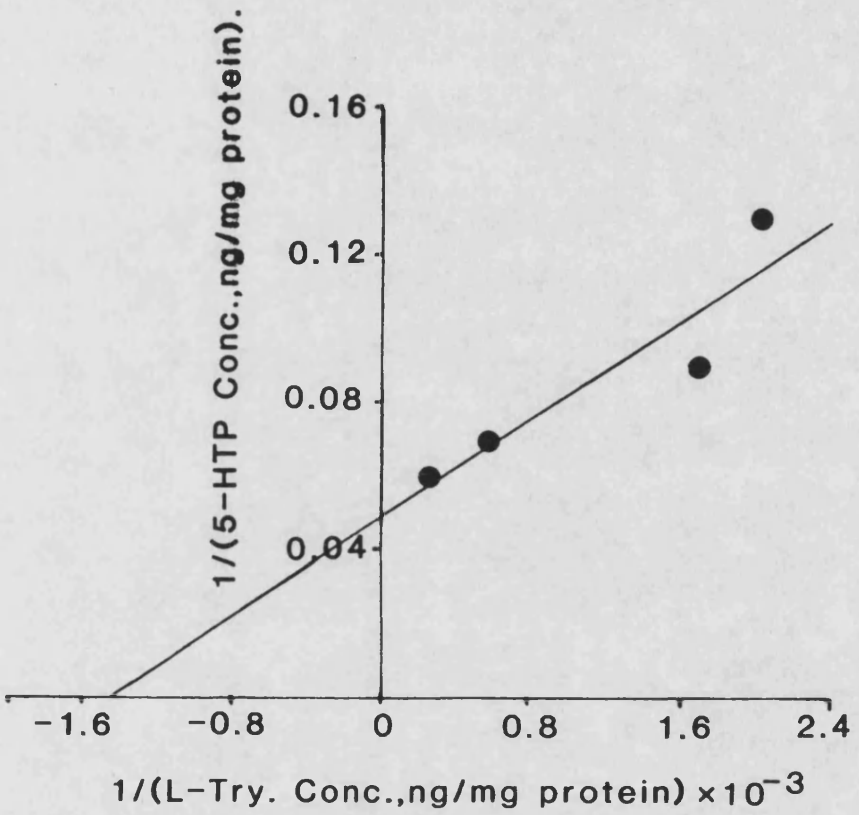


Fig.3(h).

Lineweaver-Burk plot of the effect of L-try on the in vivo activity of Try-OHase in the rat cortex. Km = 130 µM and Vmax = 0.8 µg/g of cortical tissue.

concerning the circadian rhythm of the activity of tryptophan hydroxylase measured *ex vivo*.

At this stage, having obtained evidence for a circadian rhythm of 5HT synthesis, it was deemed appropriate to proceed to investigate how this rhythm responds to treatment with antidepressant drugs. This forms the subject of the next Chapter.

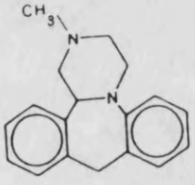
Chapter 4

4. THE EFFECTS OF ANTIDEPRESSANT DRUGS ON THE CIRCADIAN RHYTHM OF 5HT SYNTHESIS

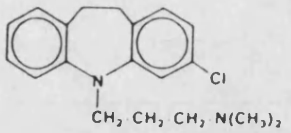
4.1. INTRODUCTION

The pathological disorders and malfunctions of neurotransmitter systems that are associated with affective illnesses, together with the rationale for application of antidepressant treatment have been discussed in chapter one. Before proceeding to determine the effects of antidepressant drugs on the circadian rhythm of 5HT synthesis, I think it is pertinent here to review some of the available information on what is currently understood about the effect of these drugs on the function of serotonergic neurons. For obvious reasons, a short summary can only be presented here and the interested reader is referred to some of the excellent reviews quoted herein.

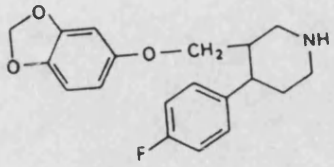
The effects of mianserin, clomipramine and paroxetine on the 5HT synthesis are examined in this study. They represent different classes of antidepressant drugs and probably act by different mechanisms. Clomipramine belongs to the tricyclic group renowned for their ability to inhibit reuptake of monoamines at the nerve endings. Paroxetine is a more selective 5HT reuptake blocker. Mianserin, a tetracyclic piperazinoazepine, is on the other hand a powerful α_2-adrenergic receptor blocker with marked 5HT antagonistic properties. The chemical structures of these drugs are represented below:



Mianserin



Clomipramine



Paroxetine

The standard form of treatment for depressive illness in clinical practice involves mainly the use of antidepressant drugs and electroconvulsive therapy. The antidepressant drugs are made up of monoamine oxidase inhibitors (MAOI's), the tricyclics and the so-called "second generation" or "atypical" antidepressants. MAOI's are generally believed to act by raising the concentration of monoamines in the central nervous system due to inhibition of MAO, the main monoamine deaminating enzyme. One serious toxic reaction to MAOI's is the hypertensive crisis, the so-called "cheese reaction", which results when certain types of food that contain a high concentration of tyramine are ingested. This is because tyramine is no longer metabolised and is taken up by peripheral noradrenergic nerve endings displacing NA which produces the hypertensive crisis. This toxic reaction has been minimized by development of more selective inhibitors. For example, deprenil is a selective inhibitor of MAO type B. It does not inhibit the predominantly peripheral type A and is

therefore less likely to produce a hypertensive crisis. Nevertheless, the MAOI's have been largely replaced by other safer forms of treatment, except in cases of non-responders.

The main mechanism of inactivation of monoamines at the nerve endings is the high affinity, energy requiring uptake system (Iversen, 1975). This process is inhibited by the tricyclic antidepressants, some examples of which are: chlorimipramine, imipramine and amitryptyline. They have different potencies in inhibiting 5HT, NA and DA uptake with some considerable overlap amongst them and their metabolites as well. For example, though chlorimipramine is the most potent 5HT uptake inhibitor amongst the tricyclics, its major metabolite, desmethylchlorimipramine is an inhibitor of NA uptake.

Newer tricyclics and more specific monoamine reuptake inhibitors have been introduced, belonging to the so-called "second generation" of antidepressants. Fluoxetine, citalopram and paroxetine are more specific inhibitors of 5HT uptake (Pinder 1985). Nomifensine and maprotiline are more specific for DA and NA uptake respectively and show least activity towards serotonergic systems. Another group of the second generation, represented by mianserin and iprindole, are inactive against MAO or reuptake process. They do not show up positively in the classical pharmacological screening tests for antidepressants yet they are effective therapeutically. They have therefore been termed "atypical" antidepressants.

Lithium and electroconvulsive therapy (ECT) are other well established forms of antidepressant treatment. Lithium is one of the most researched antidepressants yet its role is still uncertain. It

is mainly used in current practice as a prophylactic agent against the manic-depressive syndrome. ECT is the oldest of the current forms of antidepressants, having been introduced in 1938 by Cerletti and Bini (quoted by Green and Constain, 1981). Repeated shocks spread over several days are necessary and it is only recently that its mechanism of action, which clearly involves monoaminergic system, is beginning to be understood.

L-Tryptophan and its hydroxylated metabolite, 5HTP have been used alone or in combination with other antidepressants and encouraging results have generally been found, at least in some forms of depressive illnesses (van Praag, 1981). This has been one of the most convincing evidence in support of the indoleamine hypothesis of affective disorders.

It is evident therefore that the different forms of antidepressant treatment all involve at least one or the other of the monoamines 5HT, DA and NA. What is not clear is which system is the most important. However, such an argument is probably irrelevant in view of the fact that all the neurotransmitter systems including the amino acid and neuropeptide co-transmitters mutually interact (Racagni and Brunello, 1984; Costa, 1982). Pertubation of one system would therefore be expected to modulate the rest and eventually the overt mood or behaviour of an individual.

Nevertheless it is difficult to resist some of the more convincing evidence which implicates serotonin as playing a major role, at least in some forms of depressions. Shopsin, Cassano and Conti (1981) surveyed literature on newer antidepressants and concluded that those agents more specific for 5HT system were consistently more successful therapeutically than those towards NA or

DA. Moreover, in one of their earlier studies, they demonstrated that PCPA, an inhibitor of 5HT synthesis, reversed the antidepressant effects of imipramine and tranylcypromine (a MAOI) while α-methyltyrosine, an inhibitor of NA did not. The indoleamine hypothesis is further supported by the moderately successful use of L-TRY and 5HTP as antidepressants (Van Praag, 1981). Because of these considerations, the rest of this chapter and indeed the whole thesis is discussed with particular attention to the serotonergic system.

4.2. EFFECTS OF ANTIDEPRESSANTS ON SEROTONERGIC SYSTEMS

The net effect of MAOI's and reuptake blockers is an increase in the concentration of monoamines available for synaptic transmission, though the same cannot be said for the "atypical" group of antidepressants. The high concentration of 5-HT at the synapse is soon counteracted by compensatory pre- and postsynaptic mechanisms which evoke changes in synthesis and release of 5HT and receptor responsiveness. Since therapeutic effects of antidepressants are observed only after repeated administration, it is important to distinguish between acute and chronic effects.

4.2.1. Acute effects of antidepressant treatment

Acute effects of antidepressants are mainly seen with the tricyclics. They have been shown to reduce the firing rate of the serotonin containing cells of the raphé nuclei (Scuvee-Morean and Dresse, 1979). This is in turn reflected in the decrease of 5HT turnover (Carlsson and Lindqvist, 1978b, quoted by Willner, 1985). On

the other hand, Mianserin and other "atypicals" which have no effect on the concentration of synaptic 5-HT do not alter its turnover. (Marco and Meek, 1979; Kafoe, De Ridder and Leonard, 1976).

Electroconvulsive shock activates 5HT neurons causing an increase in 5HT release, as measured by increased 5HIAA level in rat brain (Evans et al., 1976) and there are some reports that it decreases its uptake as well (Minchin et al., 1983). These observations would indicate an activation of the 5HT system.

The postsynaptic receptors are generally activated by the tricyclics. This is seen for example by an increase in the frequency of head twitches in rats and mice induced by low doses of 5HTP (Ogren et al., 1982, quoted by Willner, 1985) or increase in prolactin release in humans (Anderson and Cowen, 1986), a process believed to be mediated by $5HT_1$ receptors (see sect 1.1.7.e). Mianserin has no potentiating effects on 5HT neurons on acute treatment, but because of its post-synaptic receptor blockade, it antagonizes many 5HT-mediated responses (Maj et al., 1978; Jones, 1980).

4.2.2. Chronic effects of antidepressant treatment

Following chronic administration of antidepressants, some of the acute effects cited above are modified due to adaptive changes at pre- and postsynaptic receptors in an effort to restore homeostasis. These changes are probably more relevant to therapeutic response.

Sugrue (1981) and Willner (1985) have recently reviewed effects of chronic antidepressant treatment on pre- and postsynaptic 5-HT function. These authors concluded that in general, agents that reduced turnover acutely also did so chronically while those without any acute effects were likewise inactive on long term treatment.

These effects were observed in laboratory animals as well as in patients, as assessed by concentration of CSF 5HIAA.

The postsynaptic receptor response to iontophoretically applied 5HT or 5-methoxy-dimethyltryptamine (5-MeO-DMT), a 5-HT receptor agonist, is substantially enhanced in most cases by chronic antidepressant treatment. This has been observed mainly in all brain areas except the cerebral cortex (Sugrue, 1981; Willner, 1985). Behavioural responses to various 5HT agonists are enhanced, e.g. induction of head twitches by 5HTP. Similar effects are observed following repeated ECS (Evans, et al, 1976; Graham-Smith, Green and Constain, 1978).

The response of $5HT_1$ and $5HT_2$ receptors to antidepressant treatment is very inconsistent. In general, $5HT_1$ are insensitive while in most cases $5HT_2$ receptors are down-regulated, as measured by a decrease in receptor number (B_{max}) rather than by affinity change (Kd) (Willner, 1985; Ogren and Fuxe, 1985; Enna et al, 1981 and Green and Nutt, 1983). Though this would imply an involvement of $5HT_2$ receptors in antidepressant response, in view of several inconsistencies, the significance of these findings are unclear and remain to be elucidated.

One reasonable conclusion from all this analysis is that antidepressant treatment generally leads to an enhancement of transmission across the 5HT synapse either by reuptake blockade or by increasing receptor responsiveness to 5HT or its agonists (Willner, 1985; Fuller, 1981). Nevertheless, it is clear that this may be an oversimplification of a more complicated picture and that given the present limited understanding of the function of monoaminergic

neurons, the development of a unifying theory on the mechanism of action of antidepressants is far from complete.

4.3. EXPERIMENTAL SECTION

The rate of 5HT synthesis was measured by two techniques:
a) Ex vivo assay of tryptophan hydroxylase (Try-OHase) and
b) In vivo turnover of 5HT following 5-Hydroxytryptophan decarboxylase (5HTP-DC) inhibition. These methods have been described in detail in Chapters two and three respectively.

Chlorimipramine, mianserin and paroxetine were kindly donated by Beecham Pharmaceuticals.

4.3.1. Ex vivo assay of antidepressant effects on Tryptophan hydroxylase

Chlorimipramine was dissolved in normal saline while mianserin and paroxetine, because of low solubility were dissolved in distilled water and administered intraperitoneally (i.p.) in a small volume of 0.2 ml. The rats were initially entrained to LD 12:12 for two weeks before the drug administration. For acute studies, a dose of 20 mg/kg of each drug was injected to different groups of rats weighing 200 g - 300 g at either mid-dark or mid-light of the light-dark cycle. Control rats received only the vehicle. After 60 min the rats were killed by decapitation and the activity of Try-OHase assayed as before.

The rats were similarly entrained for two weeks before chronic treatment was initiated. A dose of 7.5 mg/kg of chlorimipramine and mianserin was then injected to different groups of rats twice daily for two more weeks. The rats continued to gain weight normally as

compared to the controls and it was concluded that these doses were not detrimental to their health. At the end of the treatment period they weighed 250-350 g. The animals were then killed at mid-dark or mid-light, 24 hours after the last dose and the activity of Try-OHase determined as before.

Acute effects of paroxetine on the ex vivo activity of tryptophan hydroxylase was also determined. Different concentrations of paroxetine were included in the incubation mixture for the assay of Try-OHase. This was done only at one time point of the light-dark cycle, that is, 10.00 hours.

4.3.2. Effects of antidepressants on 5HT turnover

The same doses and the method of administration of chlorimipramine, mianserin and paroxetine as above for acute and chronic treatments respectively were used to determine the effects on turnover. The rats were also in the same weight range.

For the determination of acute effects, the drugs were administered at mid-dark or mid-light as before. After 15 min, a dose of 75 mg/kg of NSD1015 was injected and the rats killed one hour later (i.e. 1hr 15 min after the antidepressant). 5HTP in different brain areas was assayed as described earlier.

After a chronic treatment period of two weeks, NSD1015 (75 mg/kg) was injected at mid-dark or mid-light, 24 hours after the last dose of the antidepressant. The rats were killed one hour after NSD 1015 and 5HTP measured in different brain areas as before.

4.4. RESULTS SECTION

4.4.1. Effects of antidepressants on ex vivo activity of Tryptophan hydroxylase

(a) Acute drug effects

The results of the effects of acute administration of mianserin, clomipramine and paroxetine (20 mg/kg each) are represented graphically in figs 4.4 (a)-(f). Two sets of experiments were done for each drug: mid-dark and mid-light with matching controls in each case. In all cases, the vertical bars represent SEM of the activity of Try-OHase, defined as total CO_2 evolved in pmol per mg wet of brainstem per 30 min. N values are indicated in the legends for each graph.

The difference between treated and control values were assessed statistically at each concentration of L-Try by Student's t-test. In all cases, there was no statistically significant difference between control and treated samples in all the concentrations of L-Try used either at mid-dark or mid-light. It was therefore concluded that acute mianserin, clomipramine or paroxetine had no effect on the activity of Try-OHase in the brainstem at the doses used in these experiments.

Similarly, inclusion of increasing concentrations of paroxetine (0.1 - 2.0 μg/ml) in the incubation media did not have any significant effect on the activity of Try-OHase when each is compared to control (with no paroxetine added) - fig. 4.4(g).

(b) Chronic drug effects

In contrast to acute treatment, chronic administration of mianserin and clomipramine (7.5 mg/kg BD x 14 days) had a profound

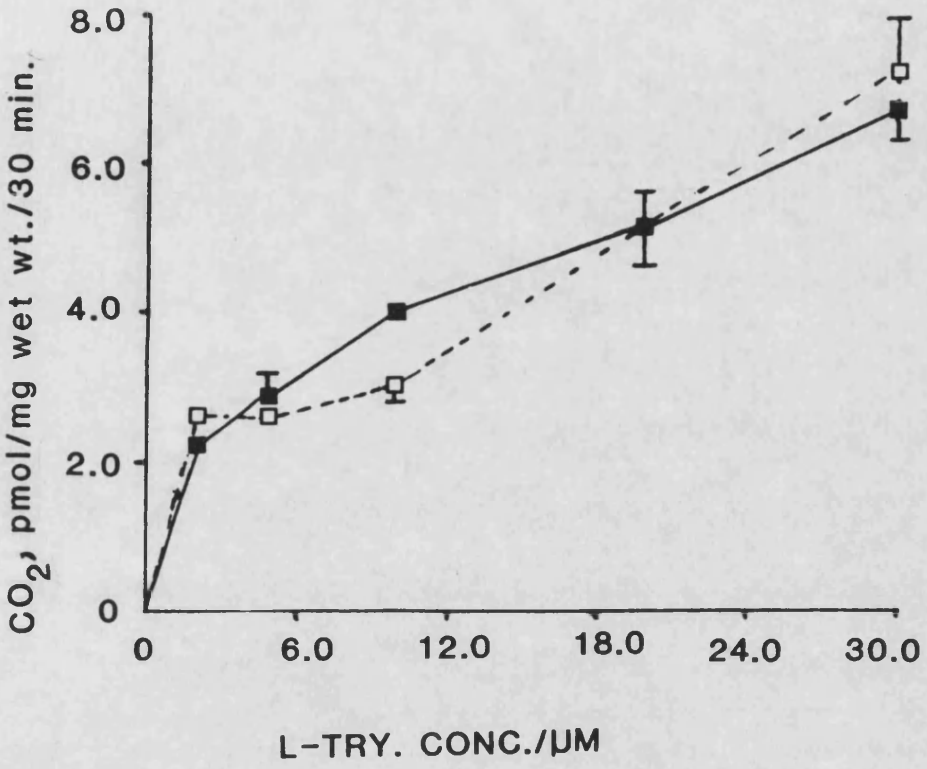


Fig. 4.4(a).

The effect of acute mianserin administration (20 mg/kg i.p.) on the activity of Try-OHase in the rat brainstem homogenates at mid-light of LD 12:12. □--------□ , treated; ■---------■ , control. The vertical bars represent the SEM of the activity of Try-OHase, defined as CO_2 evolved per mg wet wt. per 30 min; n=5.

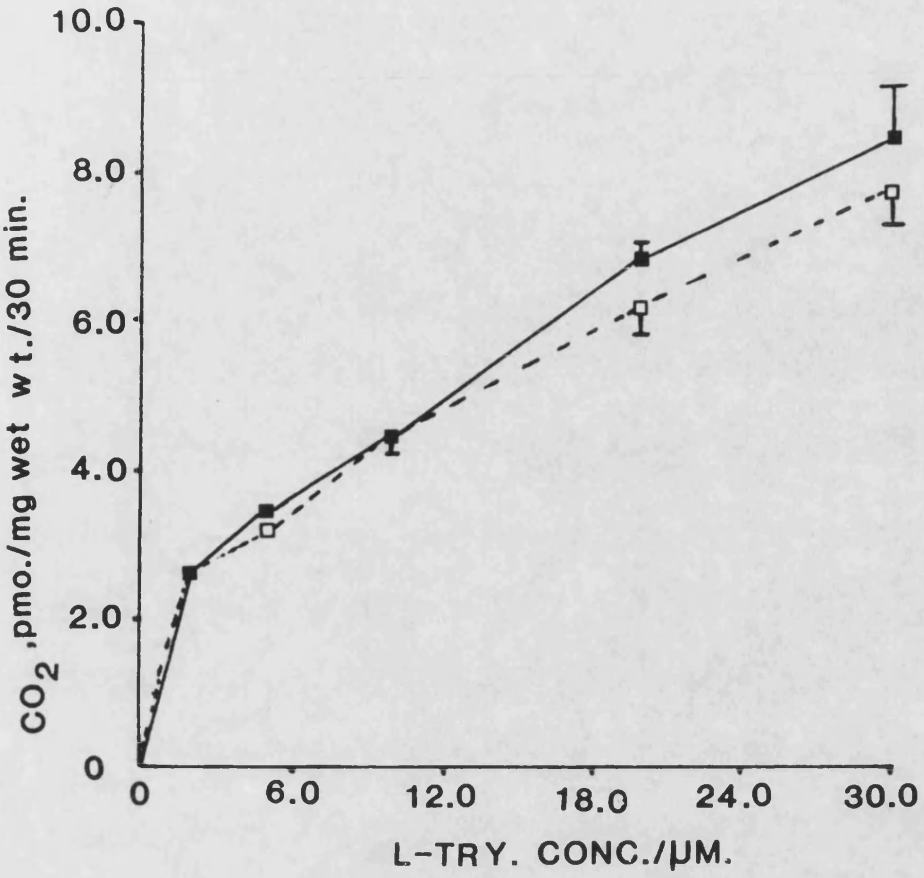


Fig.4.4(b).

The effect of acute mianserin treatment (20 mg/kg i.p.) on the activity of Try-OHase in the rat brainstem homogenates at mid-dark of LD 12:12. Treated, □- - - -□ ; control ■——■ . The vertical bars represent SEM; n=4.

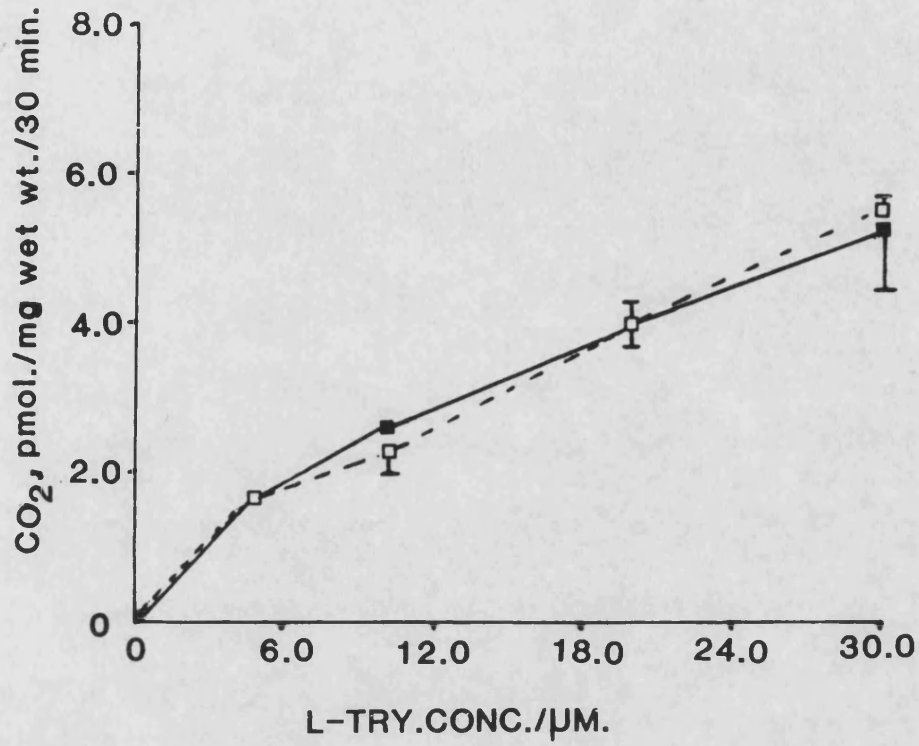


Fig. 4.4(c).

The effect of acute clomipramine (20 mg/kg i.p.) on the activity of Try-OHase in the rat brainstem homogenates at mid-light of LD 12:12. Control ■———■ ; treated □- - - -□ . The vertical bars represent SEM; n=4.

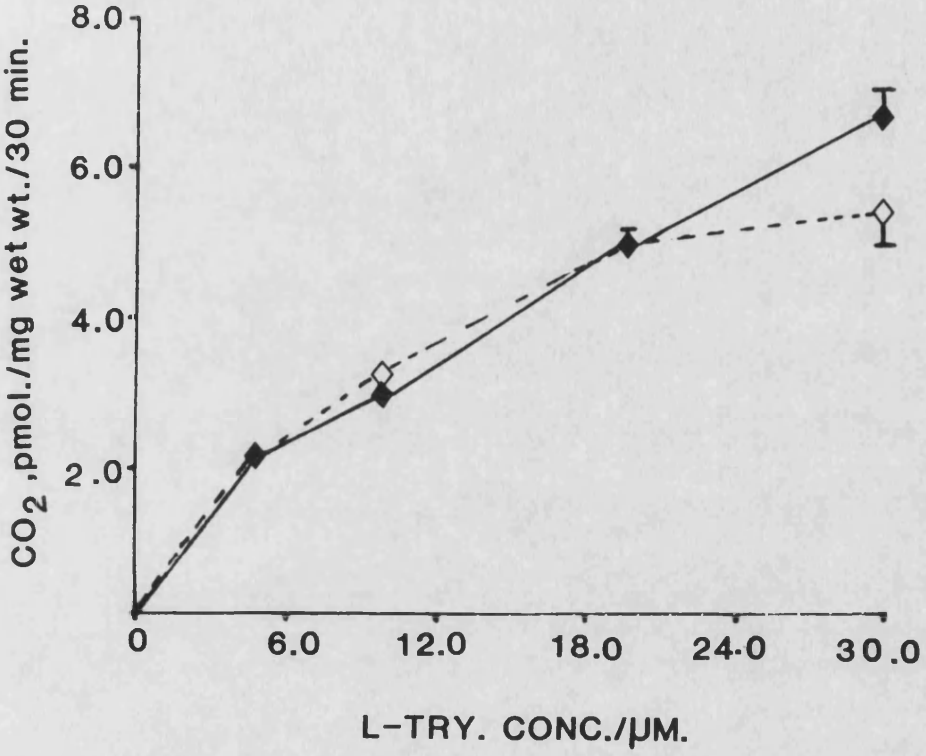


Fig. 4.4(d).

The effect of acute clomipramine (20 mg/kg i.p.) on the activity of Try-OHase in the rat brainstem homogenate at mid-dark of LD 12:12. Control, ◆——◆ ; treated, ◇---◇ . The vertical bars represent SEM; n= 4 or 5.

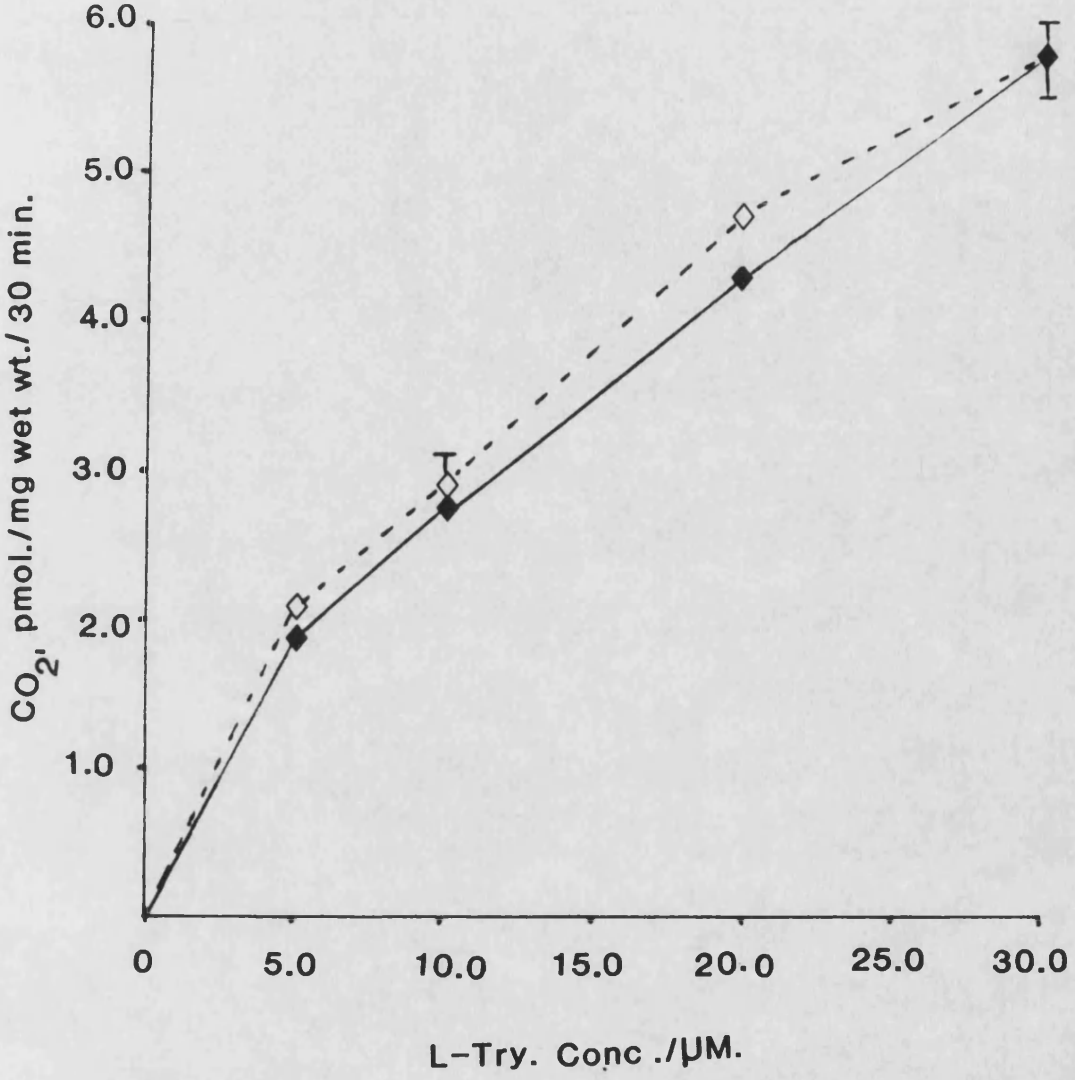


Fig. 4.4(e).

The effect of acute paroxetine administration (20 mg/kg i.p.) on the activity of Try-OHase in the rat brainstem homogenates at mid-light of LD 12:12. Control ◆——◆; treated, ◇- - - - -◇ . The vertical bars represent SEM; n=4.

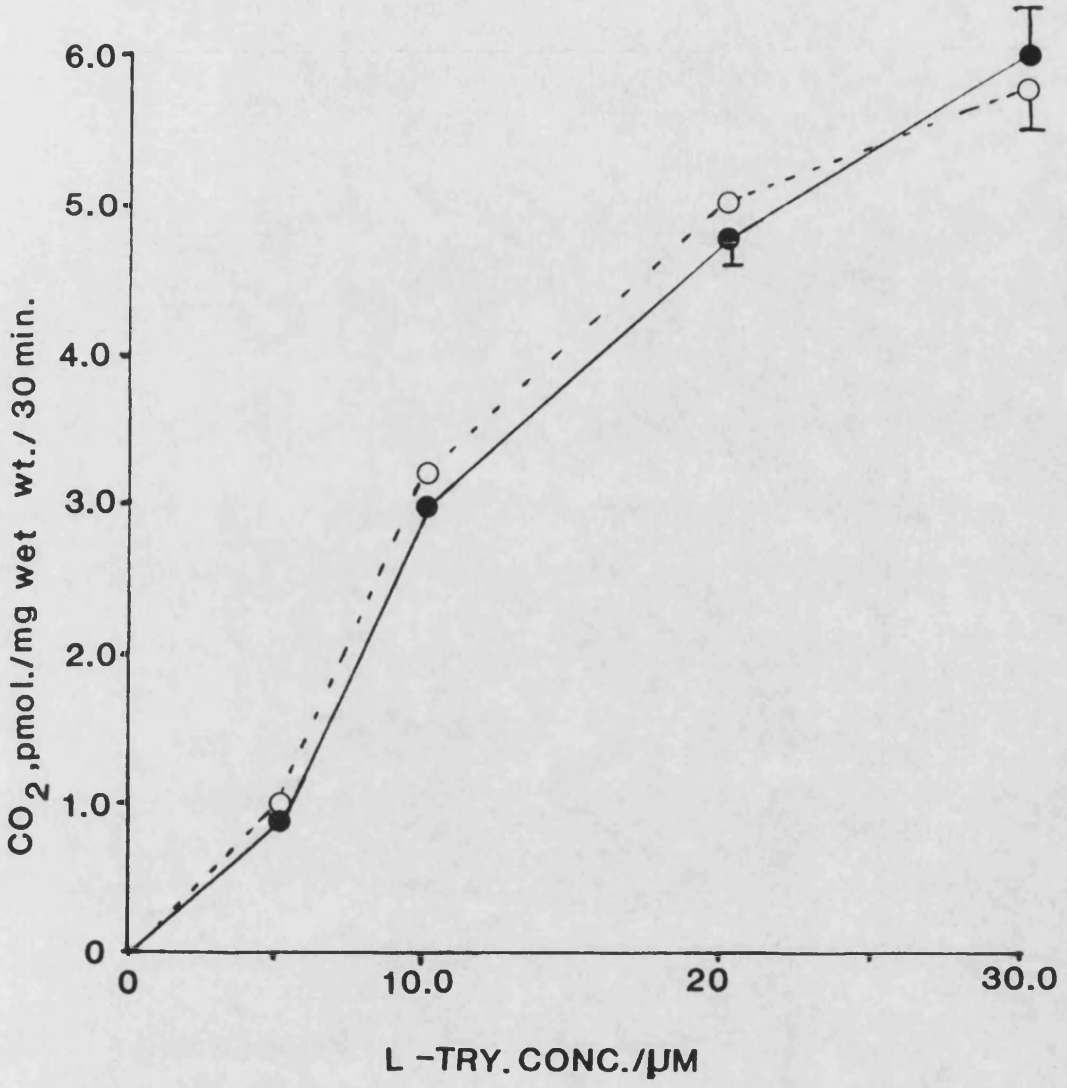


Fig. 4.4(f).

The effect of acute paroxetine (20 mg/kg i.p.) on the activity of Try-OHase in the rat brainstem homogenates at mid-dark of LD 12:12. Control, ●——● ; treated, O- - - -O . The vertical bars represent SEM; n=5.

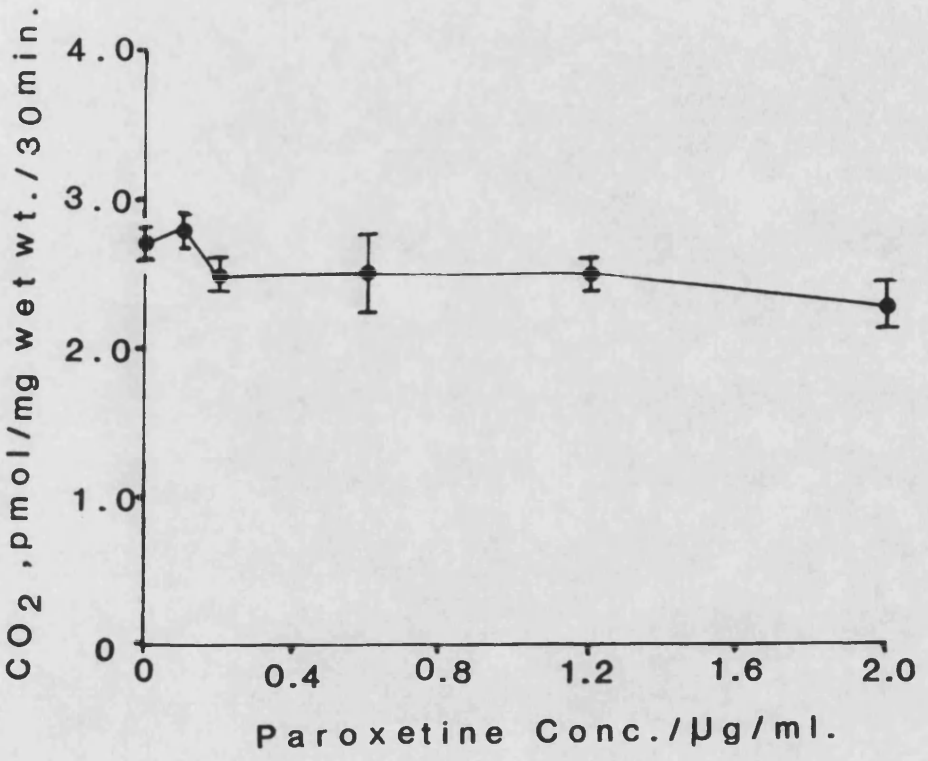


Fig. 4.4 (g).

The effect of paroxetine on the in vitro activity of Try-OHase in the brainstem homogenates at 10.00 hrs. The vertical bars represent SEM; n=3.

effect on the activity of Try-OHase. These are shown graphically in figs. 4.4(h)-(k). At least in some concentrations of L-Try, the difference between treated and control values did reach statistical significance and is indicated in the legend of each graph. It is thus clear that mianserin and clomipramine increased the activity of Try-OHase and that this effect was not different at the two time points examined, namely mid-dark and mid-light.

The activity of Try-OHase in all cases was significantly higher at mid-dark than mid-light in controls. This is in agreement with the results obtained earlier in chapter two concerning the circadian rhythm of Try-OHase activity.

4.5. EFFECT OF ANTIDEPRESSANTS ON IN VIVO 5HT TURNOVER

4.5.1. Acute drug effects

The results of acute mianserin, clomipramine and paroxetine (each 20 mg/kg) on the turnover of 5HT in the brainstem are depicted in figs. 4.5(a)-(c). The turnover is expressed as the amount of 5HTP formed in ng per mg protein of a 10% homogenate per hour. The vertical bars represent SEM and n values are indicated in each case.

Student's t-test was used to compare the treated and control values at mid-dark and mid-light for each drug. While mianserin [fig. 4.5(a)] did not induce any significant change in turnover, clomipramine significantly decreased it at both mid-dark and mid-light [fig. 4.5(b), $p<0.001$ and 0.01 respectively]. Paroxetine, on the other hand, reduced turnover only at mid-dark [fig. 4.5(c), $p<0.01$] with no significant effect at mid-light.

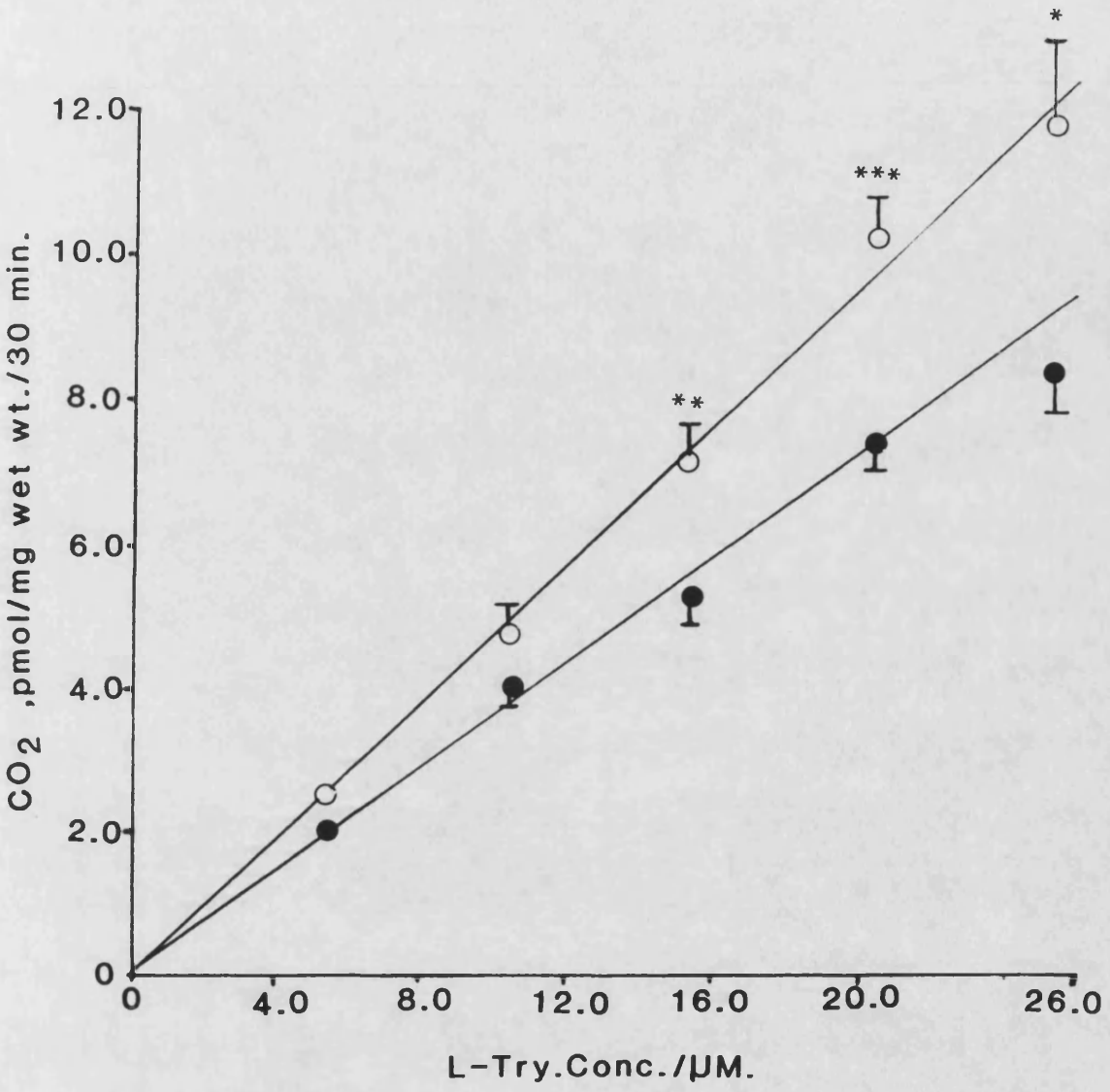


Fig. 4.4 (h).

The effect of chronic mianserin administration (7.5 mg/kg i.p. BD x 14 days) on the activity of Try-OHase in the rat brainstem homogenates at mid-light of LD 12:12. The vertical bars represent SEM. Control ●——● ; treated, O——O ; n=4. * $p<0.05$, ** $p<0.02$, *** $p<0.01$ compared to controls (Students t-test).

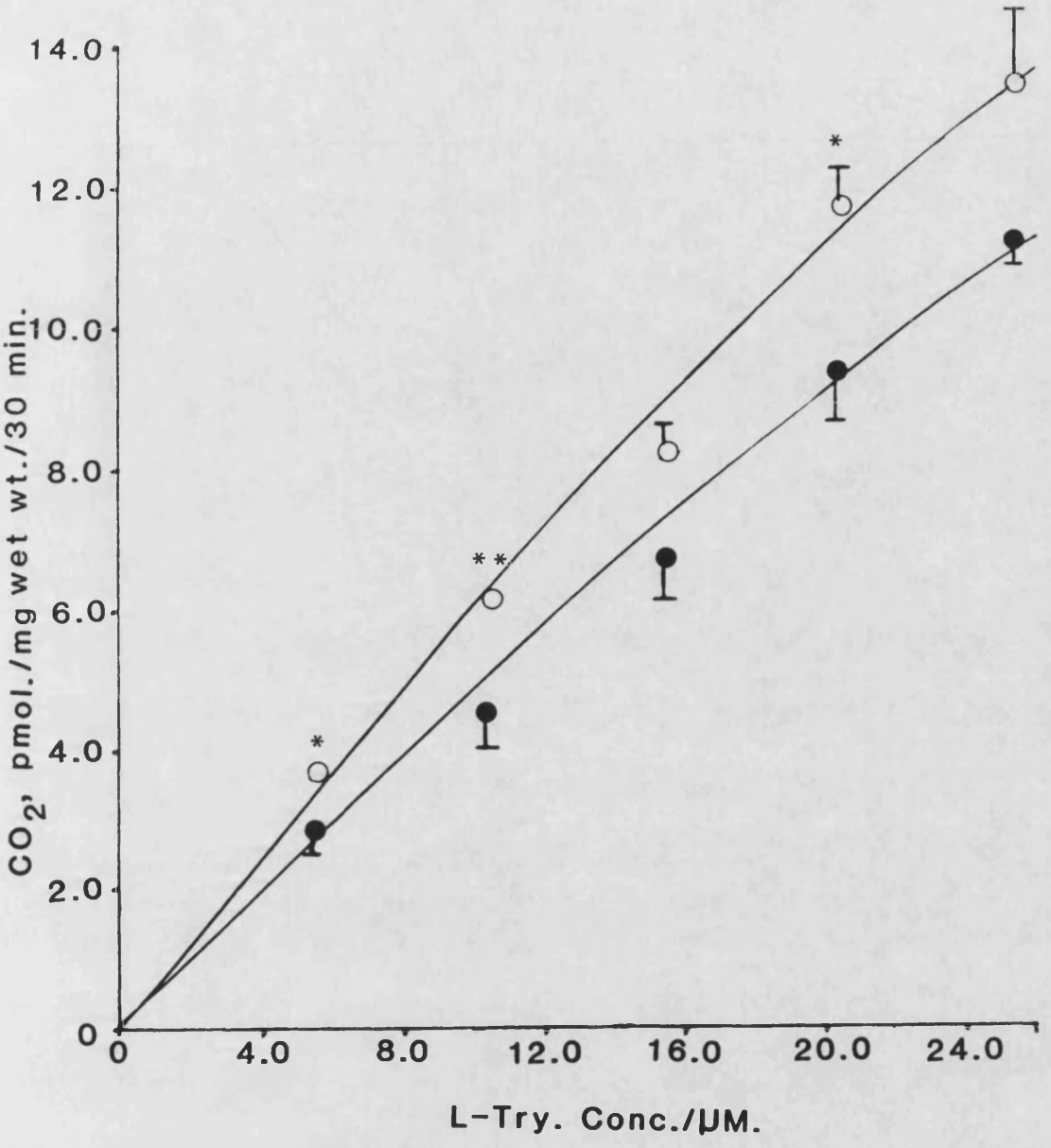


Fig. 4.4(i).

The effect of chronic mianserin administration (7.5 mg/kg i.p. BD x 14 days) on the activity of Try-OHase in the rat brainstem homogenate at mid-dark of LD 12:12. The vertical bars represent SEM; n=5. Control, ●——● ; treated, ○——○ . * $p<0.05$, ** $p<0.02$ compared to controls.

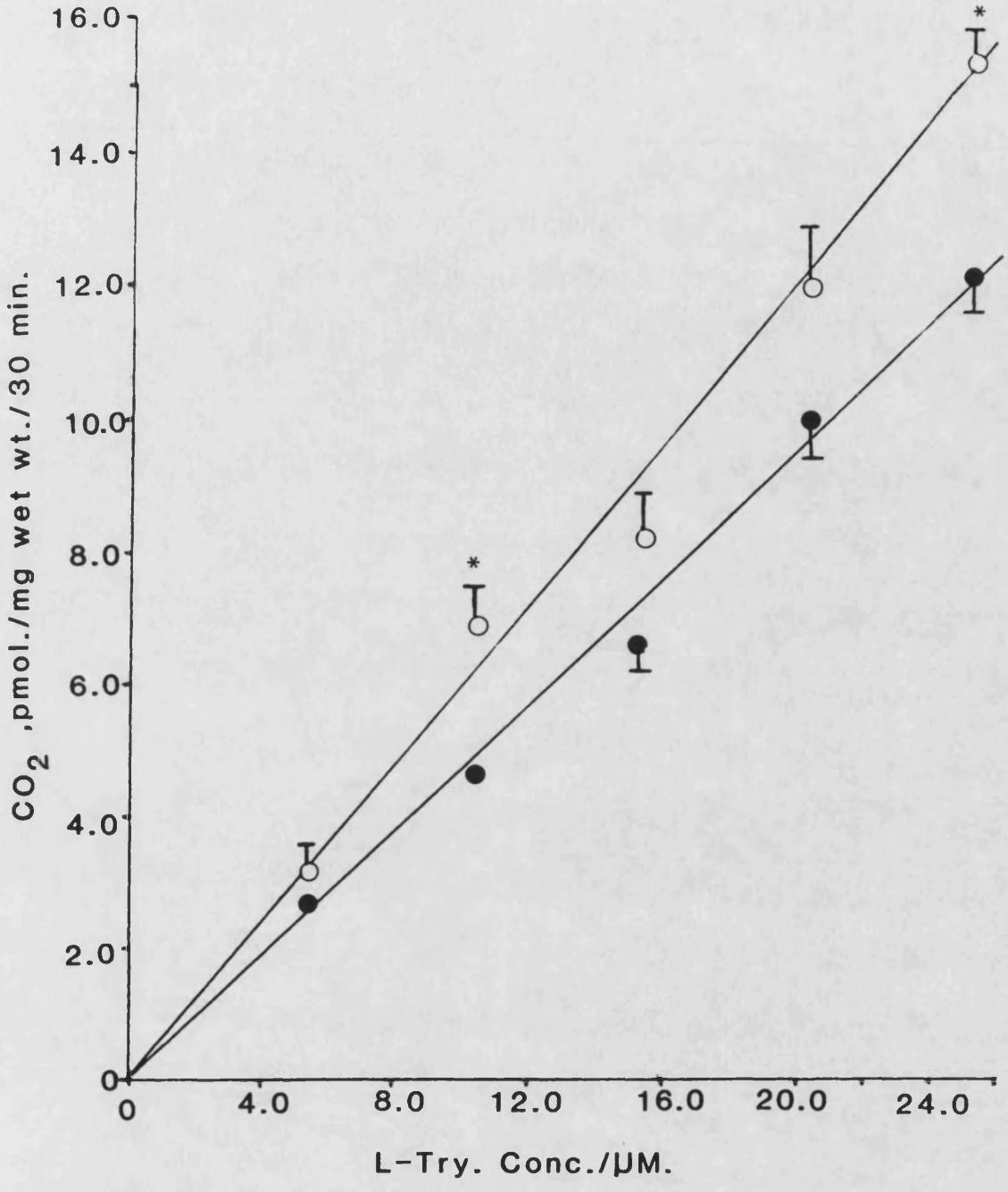


Fig. 4.4(j).

The effect of chronic clomipramine administration (7.5 mg/kg BD x 14 days, i.p.) on the activity of Try-OHase in the rat brainstem homogenates at mid-light of LD 12:12. Control ●——● ; treated, O——O . The vertical bars represent SEM; n = 3 or 4. * $p<0.01$ compared to control.

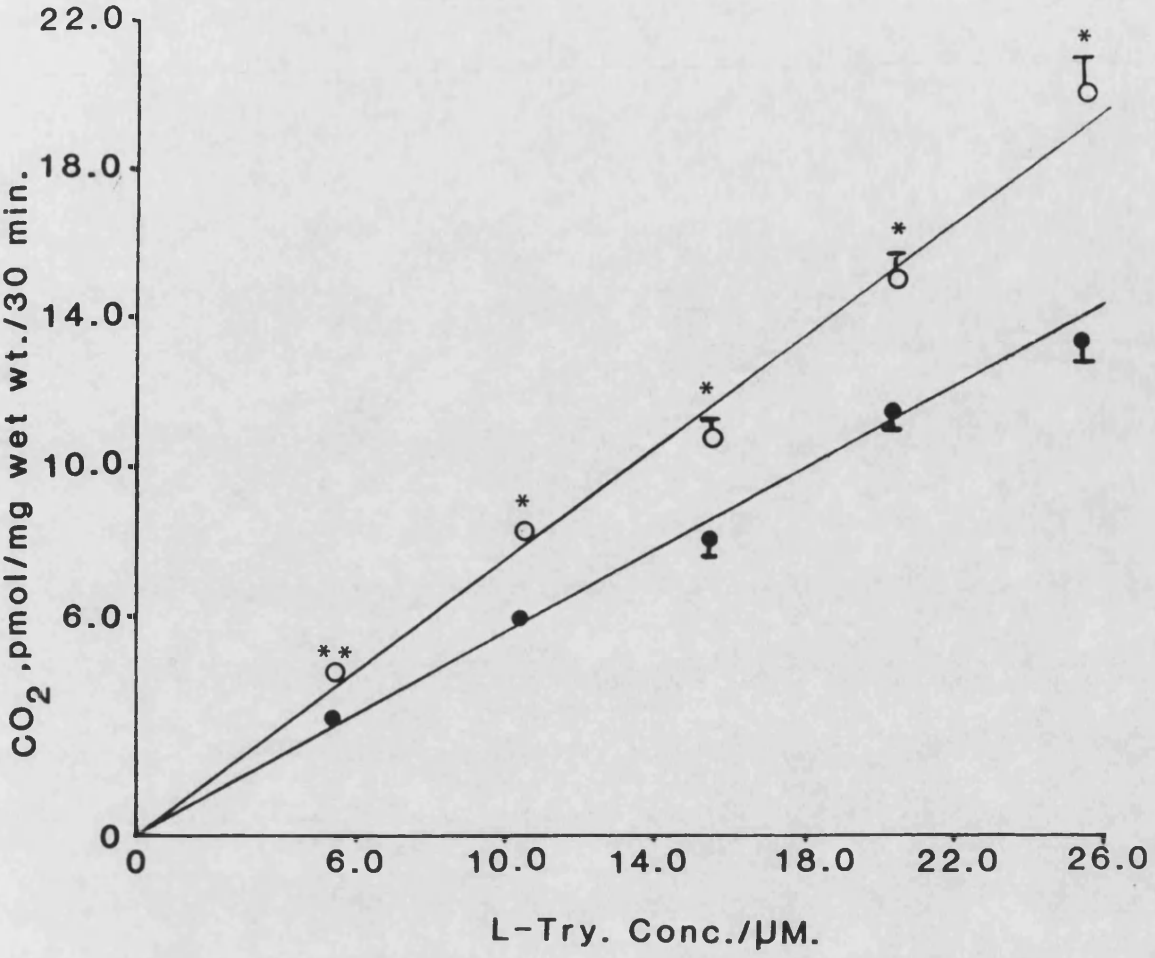


Fig. 4.4(k).

The effect of chronic clomipramine administration (7.5 mg/kg BD x 14 days) on the activity of Try-OHase in the rat brainstem homogenates at mid-dark of LD 12:12. Control, ●——● ; treated, ○——○ . The vertical bars represent SEM; n=4. * $p<0.01$, ** $p<0.001$ compared to controls (Student's t-test).

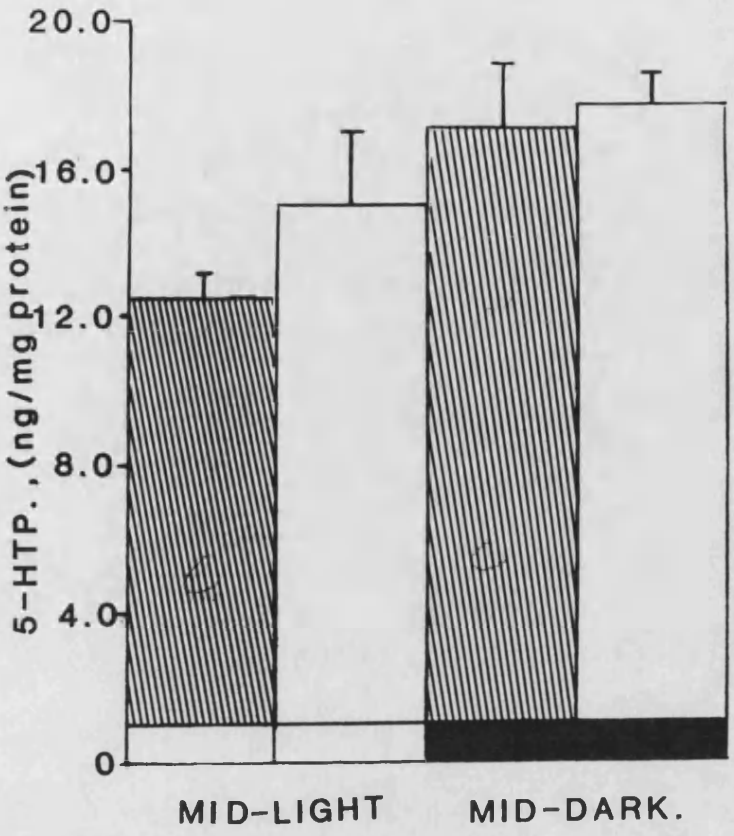


Fig. 4.5(a).

The effect of acute mianserin (20 mg/kg i.p.) on the turnover of 5HT in the rat brainstem at mid-light and mid-dark of LD 12:12. Control: hatched columns, treated: open columns. The solid black horizontal bar represents the mid-dark period; n=8.

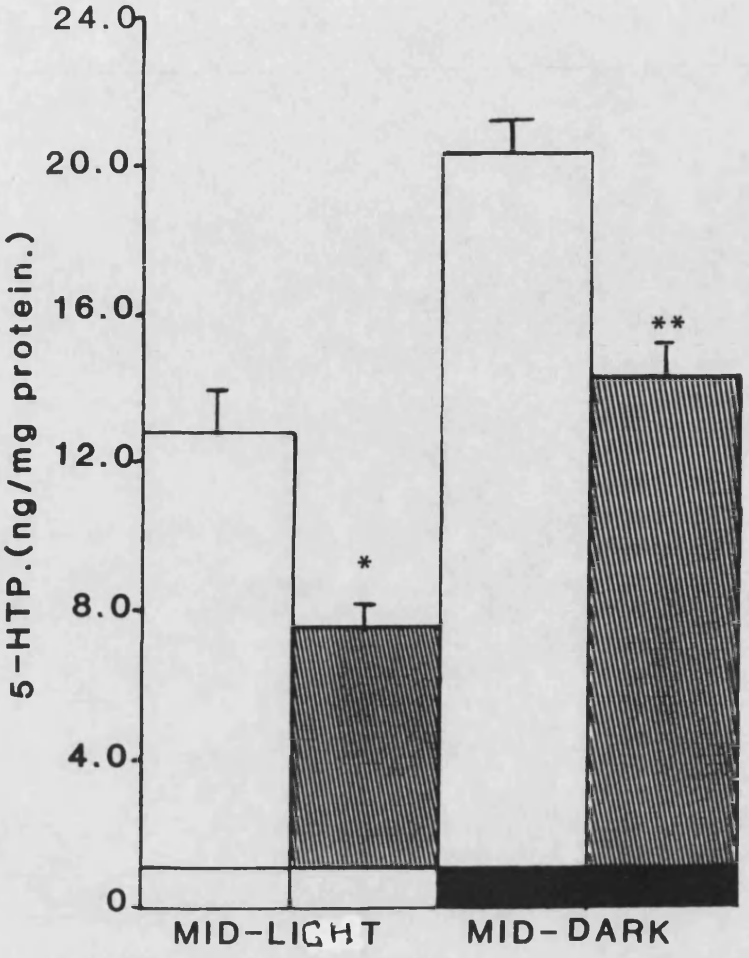


Fig. 4.5(b).

The effect of acute clomipramine (20 mg/kg i.p.) on the turnover of 5HT in the rat brainstem at mid-light and mid-dark of LD 12:12. Treated: hatched columns; control: open columns. The vertical bars represent SEM; n=6. * $p<0.01$, ** $p<0.001$ compared to controls (Student's t-test). The solid horizontal black bar represents the mid-dark period.

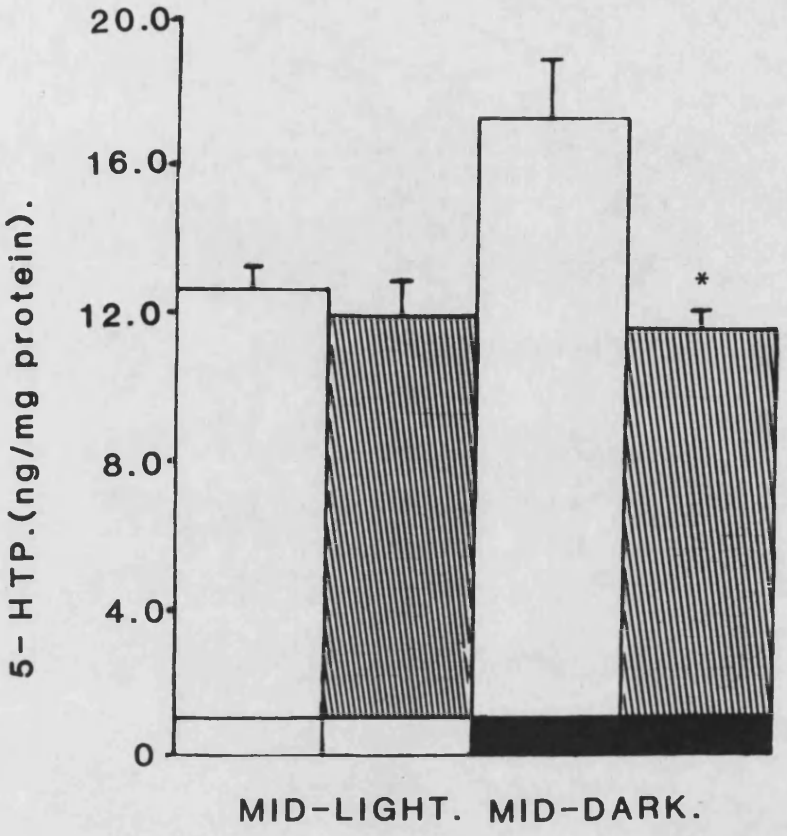


Fig. 4.5(c)

The effect of acute paroxetine (20 mg/kg i.p.) on the turnover of 5HT in the rat brainstem at mid-light and mid-dark of LD 12:12. Treated: hatched columns; control:open columns. The vertical bars represent SEM; n=7 or 8. * $p<0.01$ compared to controls (Student's t-test). The solid horizontal black bar represents the mid-dark period.

4.5.2. Chronic drug effects

Figs 4.5(d)-(h) show the effects of chronic clomipramine, mianserin and paroxetine on 5HT turnover in the brainstem and the cortex. The drugs gave different effects at the two time points. Mianserin, as seen in fig. 4.5(d), decreased turnover at both mid-dark and mid-light in the brainstem ($p<0.01$). In the cortex however, the decrease was seen only at mid-dark ($p<0.05$) but not at mid-light [fig. 4.5(e)]. The opposite was obtained with clomipramine. As seen in fig. 4.5(f), it caused a significant increase is turnover at both mid-dark and mid-light in the brainstem ($p<0.02$ and 0.01 respectively). Paroxetine generally had no effect. In the brainstem, it led to a decrease in turnover only at mid-light ($p<0.02$) but not at mid-dark [fig. 4.5(g)]. No effect could be detected on cortical turnover at any of the two time points [fig. 4.5(h)].

In all cases, the control values were significantly higher at mid-dark than mid-light, again in agreement with those obtained earlier in chapter three. The significance of these results is discussed in the next chapter.

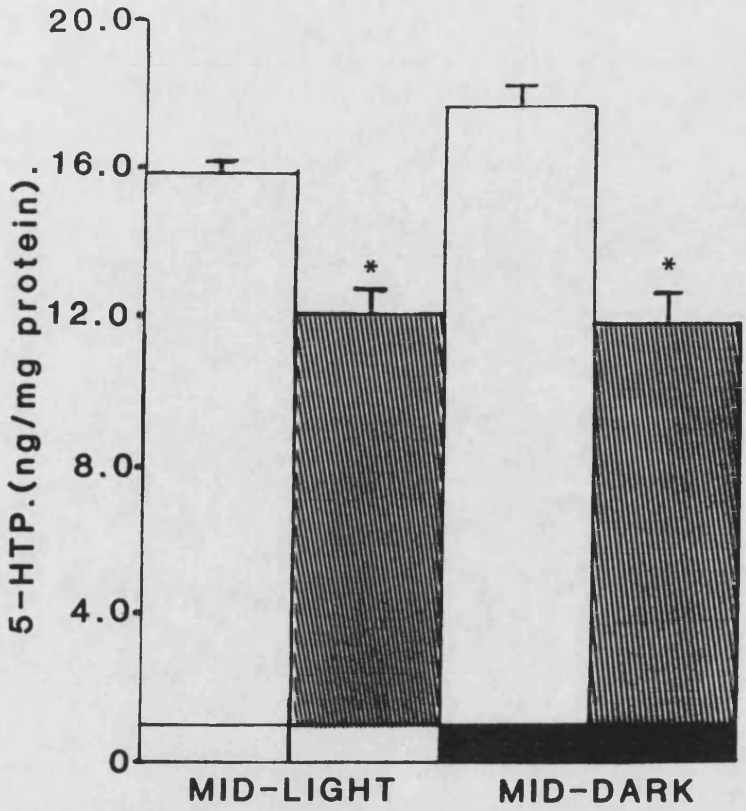


Fig. 4.5(d).

The effect of chronic mianserin administration (7.5 mg/kg BD x 14 days) on the turnover of 5HT in the rat brainstem at the mid-light and mid-dark periods of LD 12:12. Control: open columns; treated: hatched columns. * $p<0.01$ compared to controls (Student's t-test); n=4. The vertical bars represent SEM.

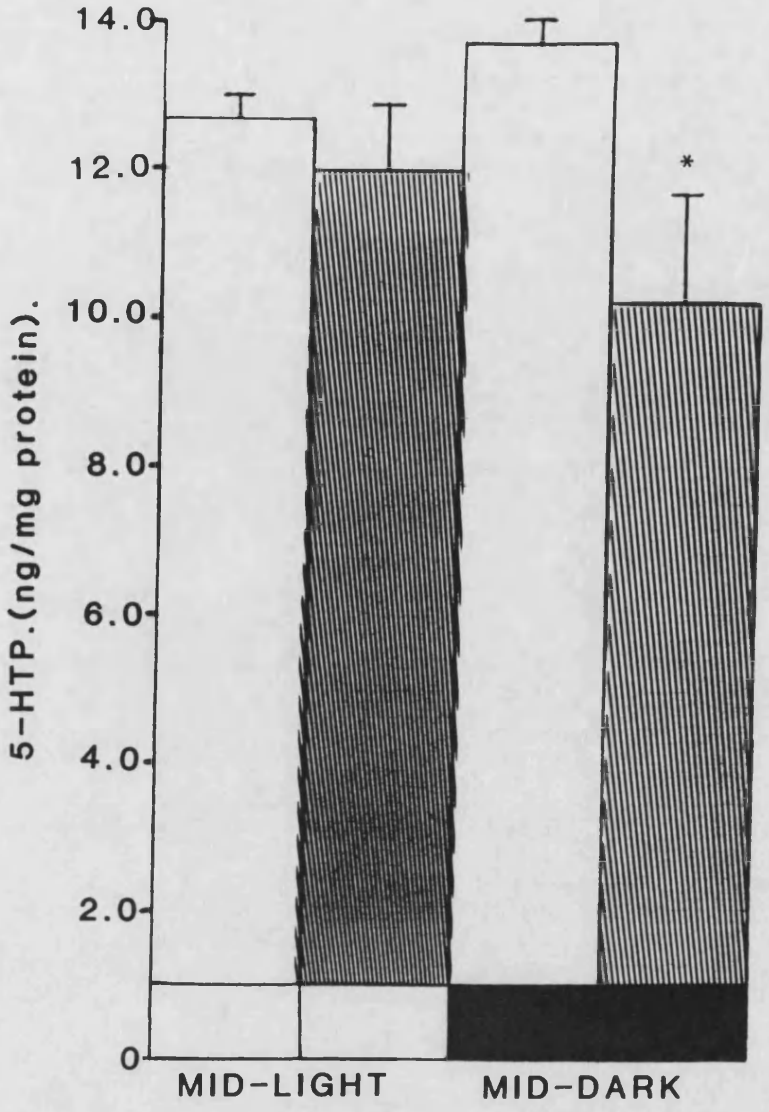


Fig 4.5(e).

The effect of chronic administration of mianserin (7.5 mg/kg BD x 14 days) on the turnover of 5HT in the rat cerebral cortex. Treated: hatched columns; control: open columns; n=4. The vertical bars represent SEM. * $p<0.05$ compared to controls (Student's t-test).

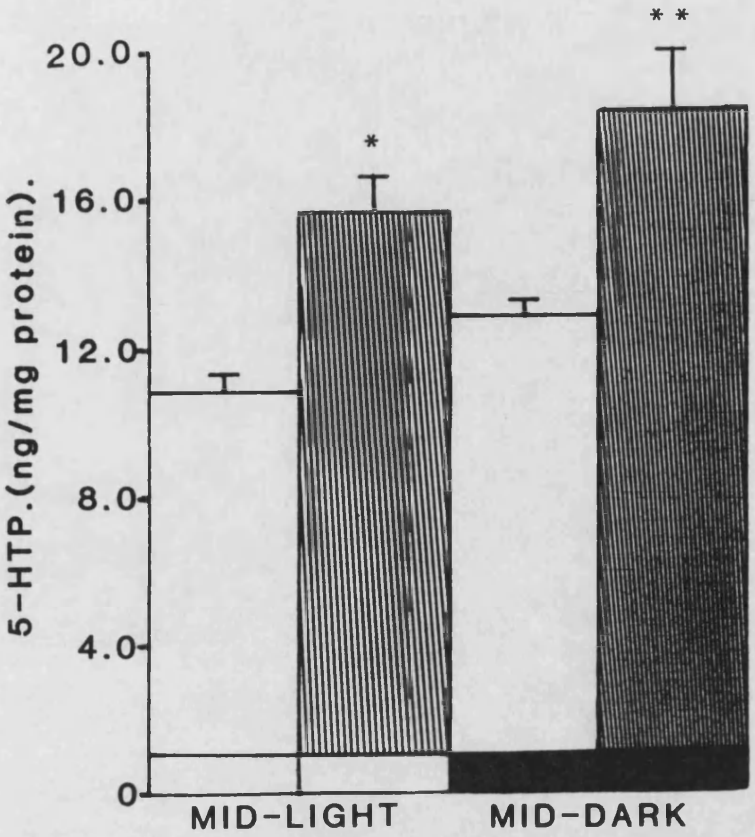


Fig. 4.5(f).

The effect of chronic clomipramine (7.5 mg/kg BD x 14 days) on the turnover of 5HT in the rat brainstem at mid-light and mid-dark of LD 12:12. Treated: hatched columns; control: open columns. The vertical bars represent SEM; n=4. ** $p<0.02$, *$p<0.01$ compared to controls (Student's t -test).

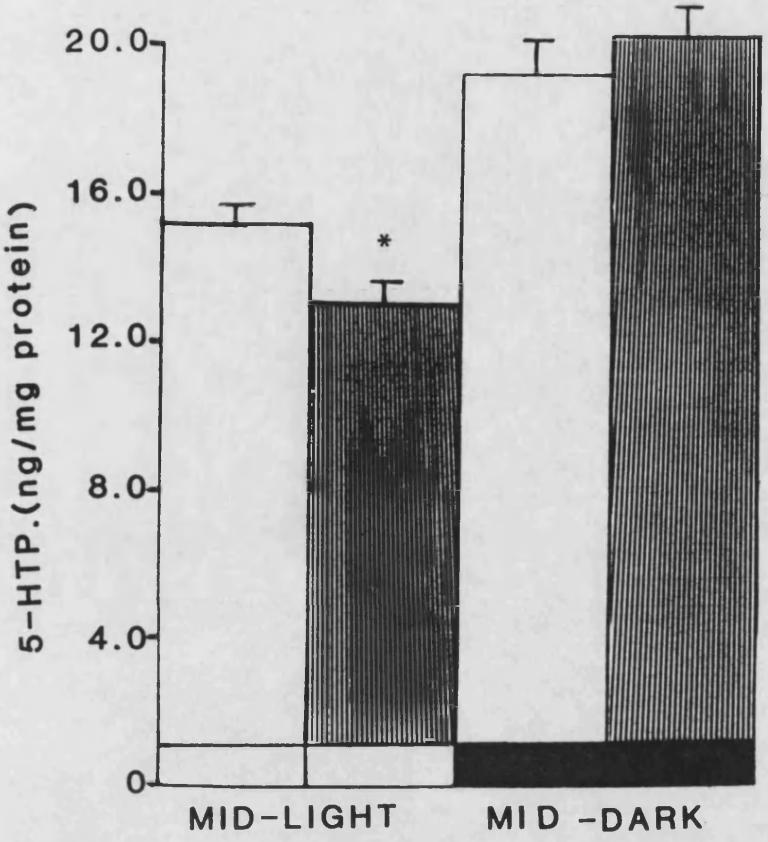


Fig. 4.5(g).

The effect of chronic paroxetine administration (10 mg/kg OD x 14 days) on the turnover of 5HT in the rat brainstem at mid-light and mid-dark of LD 12:12. The vertical bars represent SEM. Treated: hatched columns; control: open columns; n=5. * $p<0.02$ compared to controls (Student's t-test).

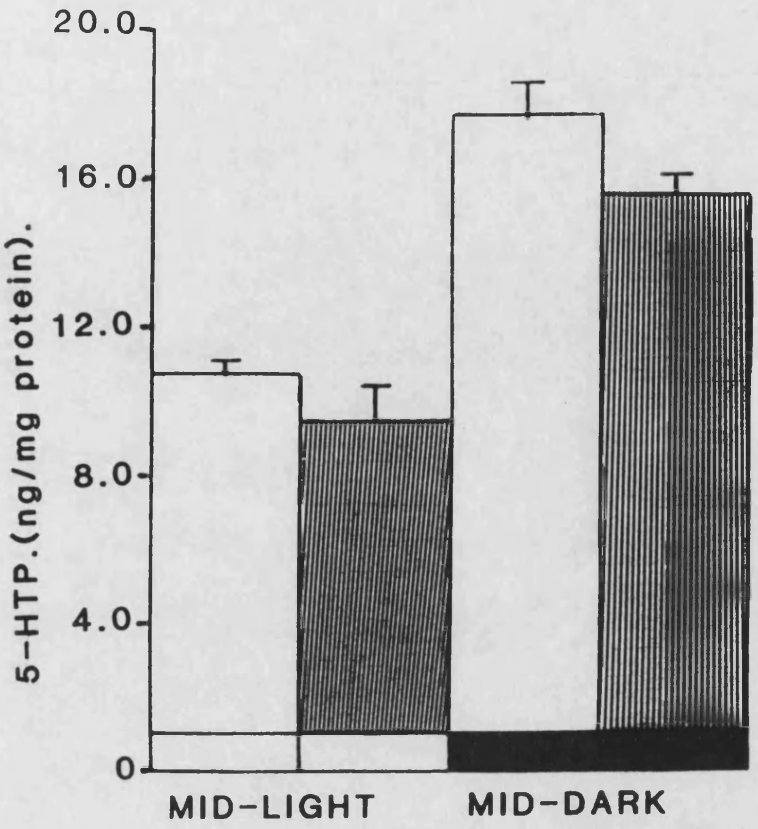


Fig. 4.5(h).

The effect of chronic paroxetine administration (10 mg/kg OD x 14 days) on the turnover of 5HT in the rat cerebral cortex at mid-light and mid-dark of LD 12:12. Treated: hatched columns; control: open columns. The vertical bars represent SEM; n=5.

Chapter 5

DISCUSSION

5.1. REGULATION OF CIRCADIAN RHYTHM OF TRYPTOPHAN HYDROXYLASE

The brainstem has a higher concentration of serotonergic cell bodies and much less terminals than most other areas of the brain. It is not surprising therefore that relatively less activity of Try-OHase than would be otherwise expected was measurable in the P_2 fraction (mainly rich in nerve endings) prepared from the brainstem than the striatum, though it was still higher than that in the cortical preparation (Fig. 2f). When the activity was measured *in vivo* however, the brainstem had a much higher ability to synthesise 5HTP than the cortex, thus confirming that a higher activity of Try-OHase resides in the cell bodies than in the nerve endings (Fig. 3f).

Ex vivo or *in vitro* assay of Try-OHase requires the addition of L-Try in the incubation medium containing the enzyme preparation and appropriate ingredients and pH necessary for optimum activity. The synaptosomal preparation (used in these experiments) first takes up the exogenous L-Try by an energy- and temperature-dependent process. This mechanism is important for determining the concentration of L-Try in the vicinity of the enzyme in the synaptosomes. It has been shown that the uptake is a rapid process and that an equilibrium is attained within 10 mins (Graham-Smith and Parfit, 1970). The incubations have been carried out here for at least 30 min and it is hoped that interferences from uptake process is therefore minimised or avoided.

The activity of Try-OHase displayed a significant circadian rhythm in the brain areas examined, namely the brainstem, cortex and the striatum. The phase relationship was similar in all areas. In all cases the activity was higher in the dark than in the light period. As can be seen in fig. 2(f) the highest activity in the

brainstem and cortex was found towards the end of the dark and lowest at the end of the light phase. The activity in the striatum generally followed a similar pattern. These results are in good agreement with those published by Cahill and Ehret (1981) who found a similar rhythm in the rat brainstem supernatant fraction prepared from high speed (30,000 xg) centrifugation of brainstem homogenates. The activity rhythm of the rat pineal Try-OHase reported by Shibuya, Toru and Watanabe (1978) is also very similar.

The results from turnover experiments (though not strictly measuring circadian rhythm) show that in vivo turnover of 5HT is higher in the dark than light period both in the brainstem and the cortex (fig 3d). Similar results were obtained by King, Steinlechner and Steger (1985) in rat mediobasal hypothalamus using a similar method of assay to the one used here. Hutson, Sarna and Curzon (1984), by sampling CSF for 5HIAA accumulation after probenecid administration, and Martin and Marsden (1985) using in vivo voltammetry to measure concentration of 5HIAA have also reported that these values are higher in the dark period.

Since Try-OHase in the rate limiting enzyme, it is reasonable to assume that the higher activity observed in the dark represents an an increased rate of 5HT synthesis, provided that the availability of L-Try, $[O_2]$ or BH_4 co-substrates are not limiting. L-Try is unlikely to be limiting at this point in the light-dark cycle because, as reviewed earlier (section 2.1), it has been repeatedly shown that its concentration is highest in the dark. The position with the other two co-substrates, $[O_2]$ and BH_4, is however not clear. These results, taken together with those obtained by in vivo assays would strongly

suggest that the rate of 5HT synthesis is higher in the dark and one would therefore expect to find a higher concentration of 5HT at this period or immediately after. On the contrary, as cited earlier in the introduction, evidence does not support this view.

Try-OHase is an enzyme which by nature has a very low activity. This has been one of the main factors which hampered its study in the past before the advent of more sensitive techniques. Its ability to convert L-Try to 5HTP *in vivo* or *ex vivo* in synaptosomes or brain slices is much less than would be predicted from the activity of Try-OHase measured *in vitro* in supernatant fractions in the presence of saturating concentrations of co-factor. In the dorsal raphe the difference exceeds two orders of magnitude (Meek and Lofstrandh, 1976). This indicates that the activity of Try-OHase in serotonergic neurons is severely restricted; and this has been attributed to the presence of an endogenous inhibitor (Mandell, 1975 quoted by McLennan and Lees, 1978). The low activity coupled with the small difference in amplitude between the zenith and nadir of the enzymatic activity is probably a major factor contributing to the controversy as to whether or not it has a circadian rhythm. The rhythm could easily be missed, especially if the technique chosen for assay is not a very sensitive one. Some of the investigators who reported negative results have determined the activity in enzymatic preparations from the whole brain. Though the results found here suggest that the phase of the rhythm is similar in the largest brain areas examined (cortex, brainstem and striatum) the activity in other areas not included e.g. the hypothalamus, cerebellum and olfactory lobes may not coincide so closely. This probably also explains the lack of any significant difference in turnover of 5HT between mid-day and

mid-night in the whole brain (table 3). Secondly, some areas such as the cerebellum or corpus callosum possess little or no activity and thus dilution factor may be considerable when such areas are included in the whole brain assays. Presumably this would tend to limit the detection of a small rhythm in a low-activity enzyme.

The intriguing question still remains to be answered: how to account for the activity rhythm of Try-OHase? That of the pineal enzyme has been more thoroughly investigated than that in the rest of the brain. Ample evidence links the involvement of the adrenergic system in this process. Interruption of the adrenergic input by use of propranolol, a β-adrenergic blocker or by bilateral ganglionectomization abolishes the circadian rhythm of pineal Try-OHase (Toru et al, 1979; Sitaram and Lees, 1984; Shibuya, Toru and Watanabe, 1978). It is not known whether a similar mechanism operates in the brain.

The availability of BH_4, L-Try and molecular oxygen, $[O_2]$ or the mechanism of their interaction with the enzyme may also contribute to the overt circadian rhythm of 5HT synthesis. As mentioned above, it is not known whether the regeneration of BH_4 or the availability of $[O_2]$ are rhythmic. Another possible contribution to the regulation of Try-OHase activity is the way the co-substrates interact with and alter the kinetics of the enzyme. Katz (1980) found that increasing concentration of L-Try in the brain led to a fall in Km of Try-OHase for O_2 and suggested that this may be an alternative mechanism by which L-Try increases 5HT synthesis without necessarily involving saturation of the enzyme.

Apart from the co-substrate factors, the circadian rhythm of 5HT

synthesis could also be due to changes in turnover of Try-OHase, i.e. its rates of synthesis and degradation. Or it may even be due to an alteration of its affinity for one or more of the co-substrates without necessarily any change in synthesis of new enzyme. Sitaram and Lees (1978) found that its activity rhythm could be abolished if the rats received cycloheximide injection 30 min before lights were switched off. They concluded that the observed increase in activity was due to synthesis of new enzyme. Toru et al., (1979) while studying the same phenomenon also reached similar conclusions. Again these data have been obtained with the pineal enzyme and it is not clear if the same holds true in the brain.

Another line of evidence suggests that changes in synthesis of Try-OHase is a very unlikely cause, to say the least, for its activity rhythm. Administration of cycloheximide (2 mg/kg i.p.) to rats 24 hours before sacrifice resulted in a loss of 20% in its activity (Renson, 1973), suggesting that it has a relatively long half-life. This is indirectly supported by the observation that a long period is necessary for total recovery after in vivo PCPA administration. A half-life of 2.5 days has been suggested for the spinal cord enzyme (Meek and Neff, 1972, quoted by Renson, 1973). If this is true then it becomes difficult to justify the claim that synthesis of new enzyme is responsible for the circadian rhythm of its activity. The synthesis of new enzyme and its axonal flow from the cell body to the terminals would both be too slow. It would appear that the most likely mechanism may involve an alteration in the affinity of the enzyme and I shall now review some evidence in support of this view.

Several lines of investigations point to the existence of a

mechanism for activation of intrinsic activity of Try-OHase *in vivo* independently of any parallel changes in the levels of L-Try or the other substrates, and that this process is related to changes in impulse flow down the serotonergic pathways. Inhibition of on-going impulses by lesions of mid-brain raphe nuclei or acute transection of tracts carrying ascending or descending 5HT fibres reduce synthesis of 5HT (Kehr and Speckenbach, 1978). In contrast, electrical stimulation of the serotonergic neurons has been shown to lead to increase in synthesis of 5HT, again without any change in L-Try levels (Herr, Gallager and Roth, 1975). However, attempts to demonstrate *in vitro* any increase in Try-OHase activity due to this treatment have been unsuccessful, probably due to a loss of the increased activity because of technical problems and the time that elapses during extraction and assay of the enzyme (Bourgoin et al, 1980, quoted by Hamon et al., 1981a,b. This problem has partly been overcome by performing experiments using brain slices or synaptosomal preparations. Incubations of these preparations either in depolarising conditions, in presence of dibutyryl cyclic AMP, Ca^{2+}, trypsin, anionic detergents or phosphorylating conditions all increase the activity of Try-OHase. However, the activity is decreased if calmodulin antagonists are included in the incubation medium. These results suggest that there is a cyclic AMP-dependent and Ca^{2+}-Calmodulin-dependent protein kinase system for activating Try-OHase (Nagatsu, Hirata and Sawada, 1985; Messripour and Clark, 1985; Hamon et al., 1981a. Boadle-Biber, 1978) and that the process may involve phosphorylation (Kuhn et al., 1980a; Yamauchi and Fujisawa 1979) leading to a loss of a regulatory polypeptide subunit (Hamon et

al., 1981a).

There is currently no evidence in the literature that this mechanism has been investigated as a possible means of regulating the circadian rhythm of Try-OHase activity. Since this mechanism can be demonstrated acutely and has been shown to occur in vivo, it is not unreasonable to assume that it may at least be partly involved. In fact recent evidence shows that cyclic AMP-dependent and Ca^{2+}-Calmodulin-dependent protein kinases co-exist with Try-OHase in the serotonergic neurons. Sawada et al, (1985) observed a significant correlation between the activities of cyclic AMP-dependent protein kinase and Try-OHase in the rat raphé and found that pre-treatment of rats with 5,7-dihydroxytryptamine led to a fall in the activities of the two enzymes. The activity Ca^{2+}-calmodulin dependent protein kinase disappeared altogether in the drug treated rats.

Furthermore, there are reports in the literature of the presence of a circadian rhythm of the firing rate of the serotonergic neurons in the CNS. Inouye and Kawamura (1979) found that multiple unit activity recorded electrophysiologically in several areas of the rat brain (e.g. caudate nucleus, dorsal and median raphé mid-brain, hippocampus etc) had a higher activity in the dark than the light period, except in the suprachiasmatic nucleus (SCN) where there was a tendency toward a daytime maximum.

On the basis of available evidence, I therefore offer the following as a speculative mechanism controlling the activity of Try-OHase: an increase in tonic electrical activity of the serotonergic neurones at the onset of darkness beyond a certain threshold activates either one or both of the two protein kinases described above, which in turn activates Try-OHase by phosphorylation. The

decrease in the impulse flow following the onset of light reverses the equilibrium, leading to dephosphorylation of Try-OHase thereby diminishing its affinity, hence its activity. This is an area which obviously requires further study.

As discussed earlier, the circadian rhythm of tissue levels of 5HT in the brain does not tally with that of its rate of synthesis. The circadian rhythm of the pineal 5HT concentration enzyme has been explained by the argument that it is a response to the rhythm of N-acetyltransferase (see section 1.1.8). No such satisfactory explanation has yet been offered for the brain 5HT rhythm, though it is most likely to be related to its rates of synthesis and utilization. Rats, mice and other rodents (from which most data have been obtained) being nocturnal animals, are mainly active in the dark. Reports abound in the literature of circadian rhythms on drinking, eating and locomotor activity which all peak in the dark. Measurement of 5HIAA by <u>in vivo</u> voltammetry, as cited above, shows that its concentration is highest in the dark and that its rhythm is superimposable on that of locomotor activity. Though the use of 5HIAA as an index of release and utilization of 5HT is complicated because it may partly result from metabolism of 5HT released in the cytoplasm (Wolf, Youdim and Kuhn, 1985; Kuhn, Wolf and Youdim, 1986) indications are that it is mainly due to synaptic release (Martin and Marsden, 1985). If these arguments are accepted to be true, they suggest that release and utilization of 5HT is greatly increased to meet the demands of awake and active animals at this time of the light-dark cycle. Thus, although its synthesis is also enhanced, it is probably outstripped by utilization. The reverse would hold

during the light when the animals are least active and mainly resting or asleep. Though the synthetic rate is decreased, its utilization is also diminished and this allows it to accumulate slowly in the storage granules, reaching its maximum level towards the end of the light phase. This argument has been suggested by Boadle-Biber (1982) and it appears to be the most attractive mechanism for generating the circadian rhythm of 5-HT levels. It is contributed to in differing degrees by synthesis, release and utilization of 5HT.

Having discussed the diurnal rhythm of 5-HT synthesis, the possible mechanism of its generation and its role in the physiological regulation of the diurnal rhythm of 5HT concentration in the CNS, I shall now proceed to deal in the next section with the effects of pharmacological manipulation with antidepressant drugs.

5.2. THE EFFECTS OF ANTIDEPRESSANTS ON THE REGULATION OF CIRCADIAN RHYTHM OF 5HT SYNTHESIS

Mixed results were obtained on the effects of antidepressants on the activity of Try-OHase and turnover of 5HT. These are described in detail in the results section. Briefly, paroxetine, clomipramine and mianserin had no effect on the activity of Try-OHase acutely, but on chronic administration, clomipramine and mianserin significantly increased the enzymatic activity at both mid-day and mid-night. The effects of both acute and chronic clomipramine on the turnover of 5HT were generally more consistent than those of either mianserin or paroxetine. What follows is an attempt to account for these effects as far as possible from the standpoint of known pharmacological actions of antidepressants on the serotonergic neurons.

5.2.1. Acute effects of antidepressants

It has been shown by use of different techniques of assay that clomipramine consistently decreases turnover of 5HT on acute administration (Marco and Meek, 1979; Van Wijk, Meisch and Korf, 1977; Neckers et al., 1977). Clomipramine, as explained earlier in Chapter Four, is a powerful reuptake blocker of 5HT and other monoamines at the nerve endings and the acute effects observed on turnover can be adequately accounted for on this basis. The raised concentration of 5HT at the synaptic cleft activates post-synaptic serotonergic receptors thereby increasing transmission along the serotonergic pathways. Since the abnormally high concentration of 5HT causes a greater bombardment on the post-synaptic receptors beyond the threshold for normal synaptic transmission, the feedback loops to the cell-body are also activated. The negative feedback is also believed to be mediated by pre-synaptic 5HT autoreceptors. The result is a decrease in the firing rate of the neurons, which in turn reduces turnover of 5HT and its release at the nerve endings. The result of acute clomipramine, which was a decrease in turnover at both mid-dark and mid-light, is consistent with this mechanism. The regulatory point at which this mechanism is exerted is most likely to be at the Try-OHase, as discussed earlier. The decrease in the firing rate of the neurons due to the negative feedback effect would be analogous to the interruption of the impulse flow, also discussed earlier. A decrease in the intrinsic activity of Try-OHase (probably a change in V_{max} or K_m) would be expected. Clomipramine has also been shown to decrease the concentration of L-Try in the forebrain (Neckers et al., 1977) and this may be another mechanism by which it reduced 5HT turnover. The observation that the decrease in turnover

occurred to the same extent at the two time points of the light/dark cycle suggests that the susceptibility of the synthetic regulatory mechanism to the effect of clomipramine is probably independent of time of day. When the activity of the enzyme was determined *in vitro* (which is expected to detect changes, if any, on its intrinsic activity) there was no noticeable difference between drug-treated and controls at both of the two times of day. This was also the case with the other two antidepressants, mianserin and paroxetine. The reasons for this are unclear, though it could be that the technique used for enzymatic assay was not sensitive enough to detect any changes. Alternatively, as has been suggested in the literature, any changes induced are probably lost during the process of assay. Incubation of paroxetine with the enzyme preparation also failed to produce any detectable effect.

Paroxetine is a more selective 5HT reuptake blocker at the nerve endings than is clomipramine and therefore the result of acute administration on turnover of 5HT is expected to be similar to those observed with clomipramine. The same feedback mechanism would be expected to apply. However, the results obtained show a significant decrease only at mid-dark. While this suggests that the dose of paroxetine used (20 mg/kg) may not have been enough to induce a decrease in turnover in midlight as well, it could also be interpreted to mean that the synthetic activity at mid-dark is more susceptible than that at midlight to the drug effect. That the same was not discernible with clomipramine is probably because the effect of a similar dose of clomipramine was greater and thus reduced turnover at both the two time points. Put another way, a given dose

of clomipramine probably exerts a more powerful effect on the serotonergic system than a similar dose of paroxetine.

The dose of mianserin administered (20 mg/kg i.p.) was without any effect on turnover at the two times of day. Reports also exist in the literature of the failure of mianserin to alter 5HT turnover on acute or chronic administration (Kafoe, De Ridder and Leonard, 1976). It is known that mianserin does not interfere with the reuptake mechanism at the nerve terminals and therefore has no effect on the concentration of 5HT or any other monoamines at the nerve endings. Since the negative feedback mechanism would not be activated, the results obtained here are consistent with this theory.

5.2.2. Effects of chronic antidepressants

As discussed earlier in section 4.2, chronic treatment with antidepressants lead to adaptation and sensitization of the serotonergic system resulting in more complicated effects on synthesis and turnover of 5HT than those observed in acute studies.

Clomipramine again gave more consistent results than the other two antidepressants. It increased turnover and the activity of Try-OHase in the brainstem. The effects were expressed without any quantitative difference between the two times of day. While several reports in the literature show that chronic clomipramine has no effect on 5HT turnover (Marco and Meek, 1979; Sugrue, 1980), some have recorded a decreased turnover (Van Wijk, Meisch and Korf, 1977). The usual explanation is that because of adaptation of receptors mediating negative feedback mechanism, the decrease in turnover observed on acute clomipramine disappears after chronic

administration. The results obtained here with chronic clomipramine suggest that this has occurred, followed by a rebound in turnover rate. The receptors have most likely been desensitized leading to a reversal of the negative feedback and hence the observed increase in 5HT turnover and activity of Try-OHase. It is indeed known that both the tricyclic and the "atypical" antidepressants desensitize 5HT autoreceptors (De Montigny, Blier and Chaput, 1984). Thus apart from increased rate of synthesis of 5HT, its release is also probably enhanced. The equilibrium would therefore be established at a higher level of 5HT concentration at the synapse, leading to an overall enhancement of transmission along the serotonergic pathways. This argument would be consistent with the monoamine deficiency hypothesis of depression.

Paroxetine decreased turnover of 5HT at mid-light in the brainstem with no effect at mid-dark. The decrease in turnover in the mid-dark observed on acute administration has disappeared, implying that there has been an adaptation of relevant receptors as explained above. These results, taken together with those of the acute effects, suggest that there may be a time of day difference in response to paroxetine administration in the brainstem. It had no significant effect in the cortex at the two times of day. It is not clear if this means that the in vivo synthesis of 5HT is regulated differently at the two brain areas. The results of chronic paroxetine are clearly different from those of clomipramine indicating that effects other than reuptake blockade, probably involving receptor affinity, number or sensitivity may be more

important. These are discussed below in connection with the effects of mianserin. These differences are not likely to result from insufficient dosage because the dose of paroxetine used (10 mg/kg OD) was chosen after it was found that 7.5 mg/kg BD led to deaths in a significant number of rats after two weeks, an event not seen with a similar dose of clomipramine.

Chronic mianserin resulted in an increase in the activity of Try-OHase in the brainstem at the two times of day. In contrast, the turnover was decreased in the same brain area. In the cortex, the decrease in turnover was detectable only at mid-dark, but not in the mid-light, again suggesting a difference in temporal response. Sugrue (1980) and Kafoe, De Ridder and Leonard (1976) have reported that chronic mianserin had no effect on 5HT turnover in the rat brain. Similarly, the same has been found in humans by measurement of the rate of accumulation of 5HIAA in CSF (Mendlewicz et al., 1982, quoted by Willner, 1985).

Mianserin is a potent blocker of α_2-autoreceptors, resulting in increased release of NA and hence a potentiation of noradrenergic neurotransmission. 5HT release is not solely under the control of 5HT autoreceptors, but is also influenced by α_2-autoreceptors located on the serotonergic neurons (Gothert and Huth 1980). Blockade of these receptors by mianserin would lead to increase in 5HT release, thereby enhancing transmission in serotonergic neurons as well. There is also evidence in the literature from behavioural and electrophysiological studies that chronic mianserin leads to supersensitivity of 5HT post-synaptic receptors (Blier, de Montigny and Tardif, 1984) and in contrast to tricyclic antidepressants, also

causes supersensitivity of pre-synaptic α_2-autoreceptors (Sugrue, 1983, quoted by Pinder, 1985). Both these effects indicate an increase in serotonergic neurotransmission. Though the results obtained from ex-vivo assay of Try-OHase would be consistent with this view, the turnover results suggest the opposite. The predominance of either of these effects would probably be dependent on the relative contributions by adaptation, downregulation or sensitization of the several pre- an post-synaptic receptors involved. It may also be noted in this context that several inconsistencies exist in reports on biochemical effects of antidepressants and other psychoactive drugs on the function of monoaminergic system. For example, acute clonidine depresses the firing rate of 5HT neurons in the locus coeruleus (Cedarbaum and Aghajanian, 1977) and reduces synthesis and turnover of 5HT in the cortex (Reinhard and Roth, 1982). But surprisingly, the same treatment increases the activity of Try-OHase measured in vitro (Weekley et al., 1985). These effects of clonidine are abolished if the rats are pre-treated with 6-hydroxydopamine (6-OHDA), suggesting that they are mediated by catecholaminergic pathways.

In conclusion therefore, the results obtained in this study have confirmed the existence of a diurnal rhythm of tryptophan hydroxylase activity and hence, presumably, the rate of 5HT synthesis in discrete areas of the rat brain. An attempt has also been made to harmonize this phenomenon with both the diurnal rhythms of 5HT concentration and the activity of 5HT neurons in the central nervous system under normal physiological conditions. The administration of

antidepressants, namely, clomipramine, mianserin and paroxetine had considerable though not necessarily similar effects on the serotonergic system, probably reflecting differences in potencies and specificities of these drugs. The determination of the drug effects was performed at only two times of day, namely, mid-dark and mid-light of LD 12:12. It is recognized that this does not claim to measure the drug effects on the circadian rhythm of 5HT synthesis as such. The reasoning was that since these two points represent the nadir and the zenith of the rhythm of the enzymatic activity, any marked effects would hopefully be more easily discernible at these points. It is therefore not possible to state whether or not the results obtained here support the hypothesis that antidepressants phase-delay circadian rhythms. It would be necessary to take samples at a much greater frequency for these effects to be detectable. This point should thus be taken into account when interpreting the results of drug effects. Secondly, it is known from clinical practice that normal subjects respond to antidepressant medication differently from depressed patients. Ideally, a model of a depressed rat should therefore be used when testing the effects of antidepressants. Several animal models have been developed (reviewed by Willner, 1984; Jesberger and Richardson, 1985). Of all these models, only the one involving lesioning of the olfactory lobes leads to biochemical, behavioural and endocrinological effects that are reversed by chronic but not acute antidepressant treatment (Jesberger and Richardson, 1986). Because of its striking similarity to human depression, these authors have concluded that this model is probably the most valid of all the ones available.

5.3. SUGGESTIONS FOR FURTHER WORK

In the course of preparation of this thesis, it became clear that some points require further elucidation. These are highlighted briefly in this section.

As concluded earlier, a major difficulty is encountered in trying to explain the cause of the circadian rhythm of tryptophan hydroxylase, and hence the rate of 5HT synthesis. Further investigation is needed to clarify whether or not there is a circadian rhythm in the availability of $[O_2]$ and the reduced form of pteridine cofactor, tetrahydrobiopterin, in the 5HT neurons or in the complex manner of interaction between tryptophan hydroxylase and its substrates. Secondly, evidence in the literature suggest that there may exist a mechanism for activation of tryptophan hydroxylase involving the spontaneous firing rate of 5HT neurons and one or more of the protein kinases. Since several biochemical steps are obviously involved in this process, a significant diurnal rhythm in any one of them may influence the final rhythm of the enzyme. These matters are only speculative at this stage and further studies in this line are therefore necessary. Thirdly, as suggested earlier, additional information vis-a-vis the phase advance hypothesis of depression can hopefully be obtained from antidepressant treatment if the enzymatic assays are performed more frequently, say at one or two hourly-intervals over 24-hour cycle. Finally, the validity of the results will be greatly strengthened if the experiments are performed using a valid animal model of depression.

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